MOLECULAR MECHANISMS BY WHICH ADAPTER PROTEIN SH2B1(beta) FACILITATES NGF-DEPENDENT NEURONAL DIFFERENTIATION

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2008

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Acknowledgements

I would like to thank my mentor, Christin Carter-Su who has taught me so many things about being a scientist. I am thankful for the freedom she gave me in my research projects, for her motivation, her kindness, and her guidance. Christy's dedication to her graduate students and employees is truly inspirational.

I would like to thank my committee members Jessica Schwartz, Gary Hammer, Anne Vojtek, and Liangyou, you are all fantastic role models for a young scientist, thank you for all your helpful suggestions and career advice.

I would especially like to thank Jessica Schwartz for being a second mentor to me. Besides all the wonderful advice she has given me during lab meetings and committee meetings, she has established an incredible CMB program. I would also like to thank Larry Argetsinger for his helpful suggestions and his meticulous troubleshooting and optimizing of the protocols in our lab. Larry has always been willing to help me with anything and everything. We have also had countless lunches together and I have certainly enjoyed our conversations and friendship over the years. I would like to thank Cunming Duan for his mentorship and his confidence in me to do research independently. I also wish to acknowledge Linyi Chen for all the time she spent with me while I was a rotation student in the Carter-Su laboratory. Linyi is a fantastic scientist and a tireless worker, I am indebted to her for all her advice time, thank you. I would like to thank the members, past and present of the Carter-Su lab, the Schwartz lab, and the Menon lab for their contributions to my project, for their support and for their friendship: Ram Menon, Maria Diakonova, Xiaohong Wang, Karen O'Brien, Anna Mazurkiewicz-Munoz, Balji Deo, Tracy Cui, Graciela Piwien Pilipuk, Jeff Huo, Julianne Kaplani, and the work study students Beth, Graham, Nisha, Matt Lee, Matt McElliot, Brett, Corey, and Alison. I would especially like to

thank my good friends in MSII: Jason Kurzer, Nate Lanning, Erin O'Leary, Teresa Cesena (AKA Deisha), Grace Jin (AKA ifriend), Michael Doche and Joel Cline (AKA Ken Block). As if she wasn't going to make my acknowledgements anyway, Erin (AKA EOL) has recently kept me well nourished during the long days and nights of writing this dissertation and drove to and from work when I was sick, thank you. Thanks also to Nate for our weekly crazy scientific theory discussions and our daily sports conversations.

I wish to thank my friends, Paul Schulz, Doug Gnodtke, Matt Aagesen, Michael Charles, Jason Kurzer, Evan Michaels, Terry Treemarcki, and Cathy Hsu for being outstanding friends. I would like to especially like to thank my girlfriend Marianne Juarez whose support and eternal optimism has been invaluable during these six years. Lastly, I would like to thank my family, including my brother Ian, and my parents, Steve Maures and Marianne Vick, for all their love and support.

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Abstract

MOLECULAR MECHANISMS BY WHICH ADAPTER PROTEIN SH2B1(beta) FACILITATES NGF-DEPENDENT NEURONAL DIFFERENTIATION

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Nerve Growth Factor (NGF) has long been recognized as a critical factor in the survival and maintenance of sympathetic neurons. Recent findings have shown that NGF is also required for the sympathetic neuron's axonal growth and appropriate target organ innervation during development. However, the molecular mechanisms by which NGF elicits these effects are largely unknown. The ubiquitously expressed adapter protein SH2B1 binds to active NGF receptor TrkA and has been implicated in NGF-mediated differentiation and survival of sympathetic neurons. This work provides evidence that SH2B1β facilitates the NGF-dependent nuclear export of FoxO1, a pro-apoptotic transcription factor. While SH2B1 was originally thought to localize and function only at the cell membrane, more recent studies indicated that SH2B1β undergoes nucleocytoplasmic shuttling. The work described in this thesis identifies a functional nuclear localization sequence and provides evidence that nuclear cycling of SH2B1β is critical to promote NGF-mediated differentiation of the preneuronal PC12 cell line. SH2B1β was found to specifically enhance the NGF-induced transcription of a primary response gene required for neuronal differentiation, urokinase plasminogen activator receptor (uPAR). Preventing

translocation either into or out of the nucleus abolished the ability of SH2B1β to enhance the transcription of uPAR in response to NGF. Similarly, NGF-dependent neurite outgrowth was inhibited in PC12 cells stably expressing a nuclear import defective SH2B1β. Knocking down endogenous levels of SH2B1 inhibited the NGF-induced transcription of uPAR as well as NGF-dependent neurite outgrowth, suggesting that endogenous SH2B1 is required for both NGF-dependent gene expression and neurite outgrowth. TAP tagged-SH2B1β was used to identify nuclear binding partners of SH2B1, including a putative transcription factor that inhibits NGF-dependent neurite outgrowth. Taken together, these results suggest a nuclear role for SH2B1β during NGF-dependent differentiation and survival. The ability of SH2B1β to influence the subcellular localization of FoxO1 and bind to and counteract the function of a novel transcription factor raises the possibility SH2B1β cycling between the nucleus and cytoplasm is required to shuttle transcription factors into or out of the nucleus.

Chapter 1

Introduction

The central challenge in understanding the development of the nervous system is to determine the molecular mechanisms that enable neurons to extend their axons and dendrites, precisely navigate great distances in order to find their correct targets, and selectively synapse with them. The central nervous system contains over 100 billion neurons intricately connected together, each one forming, on average, over 1000 associations with other neurons (1). Understanding such a complex network is a formidable feat. However, it is of utmost importance in order to improve the treatment of neural developmental defects, neurodegenerative diseases, and spinal cord injury. Adding to the difficulty of studying neurons within the central nervous system (CNS) is the fact that the viability of the organism hinges upon its proper function. Consequently, genetic and or physical manipulations to the CNS often result in lethality. As a result, the postganglionic sympathetic neuron has become a preferred model for studying the mechanisms involved in the formation of the nervous system.

The Sympathetic Nervous System

The sympathetic nervous system is part of the autonomic nervous system (ANS), which is critical in maintaining homeostasis in the body. The majority of the functions performed by the ANS are carried out without conscious control. Postganglionic sympathetic neurons innervate almost every organ system in the body and thus regulate many physiological processes, including blood pressure, blood glucose levels, respiration, heart rate, pupil dilation, and gastrointestinal paristalsis. During an organism's resting state, the sympathetic nervous system is basally active, although, it becomes more active during times of stress. Upon activation by a stressor, the sympathetic nervous system simultaneously affects the multiple organ systems which it innervates, triggering an increase in heart rate, mobilization of glucose from the liver, dilation of pupils and bronchi, and increased sphincter tone (2). Despite these dramatic physiological effects, animals are able to survive without a sympathetic nervous system. Examples of complete surgical extirpation of the sympathetic chain (sympathectomy) have demonstrated postoperative viability, although these animals require constant temperature control and stress free environments (3).

Development of the Sympathetic Nervous System

Postganglionic sympathetic neurons develop from neural crest cells, which migrate ventrolaterally within the mesoderm around each side of the neural tube, and form the sympathetic ganglion primordia (2). At this point the cells undergo specification and acquire noradrenergic properties (2). A portion of these noradrenergic neuroblasts give rise to the sympathetic ganglia which forms a chainlike column that traverses the length of either side of the spinal cord. It is from this position that the postganglionic sympathetic neurons are faced with the challenge of projecting axons to the developing organs which they innervate. The axons must find their way through the rapidly changing environment of the embryo and often must extend great distances across a range of tissues. While

the molecular mechanisms of axon initiation are not well understood, there have been several critical discoveries which address the mechanisms mediating extension and guidance of the axon.

Proximal Axon Extension

It has long since been recognized that axonal projections from sympathetic ganglia follow the pathways of arterial vasculature in order to reach their targets (4). Although, until recently it was unclear what was directing the axons to seek out and follow the arterial tissue. Two factors, artemin (ARTN) and neurotrophin-3 (NT-3), appear to share a role in such proximal axon guidance *in vivo* (5, 6). ARTN is a member of the glial-derived neurotrophic factor (GDNF) family and signals through the receptor tyrosine kinase RET and co-receptor GFRα3 (7). Characterization of ARTN and GFRα3 deficient mice by Honma et al. (5) revealed similar defects in the sympathetic innervation of the gut. Furthermore, ARTN transcript was detected within proximity to sympathetic neuroblasts as well as along the arterial wall (5). The arterial produced ARTN was found to be a potent chemoattractant for the sympathetic neuroblast, suggesting that ARTN guides the early axonal projections to and along the arteries.

However, the ARTN$^{-/-}$ mice did not exhibit a complete loss of sympathetic innervation, suggesting that ARTN alone cannot account for proper proximal axonal guidance and elongation. As mentioned, NT-3 provides a similar role as ARTN in proximal axon extension. Interestingly, NT-3 was originally thought to mediate the survival of sympathetic neurons because mice lacking NT-3 demonstrated a 50% reduction of cells within the superior cervical ganglion (8). In a follow-up study, Kuruvilla et al. (6) confirmed the impaired sympathetic development of NT-3$^{-/-}$ mice, although they concluded that NT-3 was important for proximal axonal extension and not sympathetic neuron survival. During the early stages of sympathetic axonal growth, the NT-3$^{-/-}$ mice displayed reduced axonal projections along the vasculature as well as reduced target innervation, but did not demonstrate an increase in sympathetic neuron death compared to

WT mice (6). Only later in development, after failure to properly innervate target organs, did the sympathetic neurons in NT-3$^{-/-}$ mice undergo apoptosis (6). Similar to ARTN, NT-3 seems to signal for the proximal axon extension of sympathetic neurons, however distal axonal extension, final target innervation, and survival are dependent upon other signals.

Paradoxically, after an initial burst of proliferation, successful development of an organism's sympathetic nervous system hinges upon the orchestrated demise of half of all neurons generated during development (9). In development as well as in maintenance, the fate of sympathetic neurons is dependent upon limiting quantities of neurotrophic factors located at a neuron's target destination. While seemingly inefficient, this is a highly selective process which dictates that the number of neuronal innervations allowed to persist will be proportional to that of the amount of trophic factor produced. Neurons receiving less than sufficient amounts of neurotrophins undergo apoptosis (9, 10). Nerve growth factor (NGF), another member of the neurotrophin family of growth factors, is critical for the survival of sympathetic neurons and elicits effects through binding to the receptor tyrosine kinase TrkA. Strikingly, mice with a targeted deletion of the NGF gene display almost a complete loss of sensory and sympathetic neurons (11).

Nerve Growth Factor and Survival of Sympathetic Neurons

Due to the absolute NGF dependence of sympathetic neurons for survival, it was difficult to assess whether NGF was required for other aspects of sympathetic neuron development. In order to circumvent this requirement for NGF, a double knockout was generated lacking NGF as well as the pro-apoptotic Bax gene (12). Sensory neurons that previously died in the NGF$^{-/-}$ mice were unable to undergo apoptosis and therefore persisted in NGF$^{-/-}$/ Bax$^{-/-}$ mice (12). Although sympathetic neurons were not investigated in this study (12). Soon after, this elegant mechanism was employed to assess NGF's role during sympathetic neuron development. In stark contrast to the NGF$^{-/-}$ mice, the NGF$^{-/-}$/ Bax$^{-/-}$ double knockout mice did not exhibit excess death of sympathetic chain neurons (13). However, several of their organs displayed either impaired or

complete loss of sympathetic innervation (13). This study concluded that in addition to survival, NGF is also required for the sympathetic innervation of target organs (13).

Taken together, these results indicate that ARTN and NT-3 function in concert to mediate axon extension of sympathetic neurons to and along proximate vasculature. Separately, NGF signaling is required for target innervation, and perhaps distal extension of the axon, in addition to its established role in sympathetic neuron survival.

Molecular Mechanisms of Neutrophin-mediated Differentiation and Survival

Several of the aforementioned *in vivo* studies have demonstrated distinct developmental consequences of deletion of critical ligands and/or receptors. However, at the molecular level, it is largely unclear as to how the cell derives such specific biological responses from these signals. For example, NT-3 was previously thought to function through the interaction with its high affinity receptor TrkC, yet deletion of TrkC within mice did not mirror the defects observed in the sympathetic neurons of NT-$3^{-/-}$ mice (14). Instead, further genetic studies demonstrated that NT-3 binds to and activates the same receptor as NGF, tyrosine kinase receptor TrkA (14, 15). Moreover, in sympathetic nerve cultures, both NT-3 and NGF treatment elicit identical responses, including the autophosphorylation of TrkA as well as the activation of the Ras/Raf/MEK/ERK and PI3K/Akt pathways (6). How the cell achieves the physiological specificity of NT-3 versus NGF from the activation of the same receptor and signal transduction pathways is unknown.

Cell Culture Model of Sympathetic Neuritogenesis

Much of our current understanding regarding the molecular mechanisms of neuronal differentiation and survival has come from cell culture work. Specifically, the PC12 cell line has been the prevailing model system for elucidating the mechanisms of NGF-mediated differentiation of sympathetic neurons. Indeed, during the 28 years between the time NGF was first discovered and the first published appearance of the PC12 cell line (16), relatively little advancement had been made towards understanding the NGF response (17). As discussed previously, the sympathetic neuron's dependence upon NGF for frank survival limits the types of experiments that can be performed, and confounds the interpretation of experimental controls which do not receive NGF. Importantly, while the PC12 cell line is responsive to NGF, it can be cultured in its absence. Derived from a solid rat pheochromocytoma tumor, PC12 cells cease proliferation in response to NGF, exhibit somatic hypertrophy, and acquire neurites (16). The NGF-dependent neurite outgrowth of PC12 cells reflect morphological and biochemical changes similar to that of differentiating sympathetic neurons *in vivo*. In response to NGF, PC12 cells express several neuronal specific markers (16) and are capable of forming synapses with primary neurons (18).

The NGF-dependent transformation of the rounded nondescript PC12 cell into a fully differentiated neuron, represents an exquisite amount of molecular control. In order for the cell to undergo such dramatic morphological and biochemical changes, a cell must specifically activate transcription of genes that facilitate differentiation, as well as repress transcription of genes that oppose the change. Utilizing the PC12 model system, many of the molecular pathways responsible for these changes have been elucidated. Early work characterizing the pathways elicited by NGF's association and activation of TrkA came to a

troubling conclusion; the pathways were identical to those elicited in response to epidermal growth factor (EGF) activation of EGF receptor (EGFR). In response to both NGF and EGF stimulation, the PC12 cell activates phospholipase $C\gamma$, PI3K/Akt, Ras/Raf/MEK extracellular regulated kinases (ERKs) 1 and 2, Jun N-terminal kinase (JNK), p38, and atypical protein kinase C (19-21). Intriguingly, these ligands facilitate very different biological responses. While EGF induces a mitogenic response in PC12 cells (22), NGF causes cessation of cell division and promotes morphological differentiation (16, 23). In the years since the identification of this phenomenon, there have been several published observations that NGF elicits a more prolonged activation of ERK than EGF. This led to the hypothesis that activation of different pathways are not required, instead, sustained verses short-lived activation of ERKs mediate either mitogenic or neurogenic effects, respectively (24-27). Indeed, the activation of ERK is required for NGF-mediated differentiation in PC12 cells (28-30). However, the identification of adapter and/or scaffolding proteins recruited specifically to activated TrkA have been identified. The scaffolding protein FRS2 recruits a complex of proteins, including Crk, C3G, Rap1 and B-Raf to active TrkA, but not active EGFR (31), resulting in the prolonged activation of ERKs 1 and 2 (32). While the duration of ERK activity does appear to be required for the differential effects of NGF and EGF, the current belief is that separate pathways are induced through receptor specific adapter/scaffold proteins, which result in different durations of ERK activity.

From this work emerged the importance of adapter proteins in the cell's ability to translate extracellular signals into specific and appropriate biological responses. Indeed, the earlier discussed issue concerning the specificity that arises from NT-3 and NGF is most likely explained by a difference in each ligand's capacity to form specific receptor/adapter signaling complexes. Potentially, this difference could be due to NT-3 and NGF causing unique conformational changes in TrkA, which form unique binding sites for specific adapter proteins. Given that NT-3 is thought to function earlier than NGF in the

developing sympathetic neuron (2), it is also possible that temporally regulated expression of specialized TrkA adapter proteins mediate the specific responses of NT-3 and NGF. In order to uncover the molecular mechanisms by which this specificity arises, the continued identification and characterization of the adapter proteins that interact with TrkA is required. To this end, the Carter-Su lab has identified the adapter protein, SH2B1β, which associates with active TrkA and enhances NGF-dependent differentiation of PC12 cells (33). The mechanism by which SH2B1β facilitates this effect is of great interest, because SH2B1β is able to associate with both active Trks (NT-3 and NGF) (33, 34) as well as RET (receptor for ARTN and GDNF) (35).

The Adapter Protein SH2B1

Cells have evolved processes by which external environmental stimuli are converted into biological responses. The process of conducting the information held by biological messengers, such as hormones and growth factors, across the cell membrane frequently involves receptors that recognize the initial signal, and molecules within the cell that transduce that signal into a cellular response. To achieve the specific and appropriate cellular responses from the signal, the cell often employs a class of proteins termed adapter proteins. Adapter proteins are important signaling molecules that usually lack intrinsic enzymatic activity, but instead serve to connect proteins together. In the case of receptor tyrosine kinases or receptor–associated tyrosine kinases [e.g. Janus kinases (JAKs)], ligand binding to the receptor initiates changes within the receptor, which activates the receptor or the receptor-associated tyrosine kinase, respectively, leading to the tyrosyl phosphorylation of the kinase and the receptor. The phosphorylation of receptors and receptor-associated kinases provides binding sites for adapter proteins that contain phosphotyrosine-binding motifs such as Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Adapter proteins are typically composed of several modular protein-protein interaction domains, allowing these proteins to link together multiple regulator and effector proteins. The many domains within adapter proteins and the specificity possessed by these domains provide these adapter molecules with the ability to elicit diverse, individual responses to a particular signal.

One group of adapters that plays a role in a variety of signaling pathways is the SH2B family of adapter proteins (FIG 1.1). Indeed, SH2B family members have been implicated in signal transduction processes for a number of receptor tyrosine kinases, including the receptors for nerve growth factor (NGF) (33, 34), insulin (36, 37), insulin-like growth factor I (IGF-I) (38), brain derived neurotrophic

factor (BDNF) (34), glial-cell-line-derived neurotrophic factor (GDNF) (35), platelet-derived growth factor (PDGF) (39), and fibroblast growth factor (FGF) (40) as well as for the JAK family of tyrosine kinases (41) (42). SH2B family members include SH2B1 (originally named SH2-B, also known as PSM), SH2B2 (originally named APS) and SH2B3 (originally named Lnk) (FIG 1.1). These proteins all share a common domain structure that includes a SH2 domain, a PH domain, several proline rich regions, and a dimerization domain. The three members of the SH2B family were first identified as signaling molecules involved in immune cell activation (43-45). After its initial identification, *SH2B1* transcript was later discovered to undergo alternative

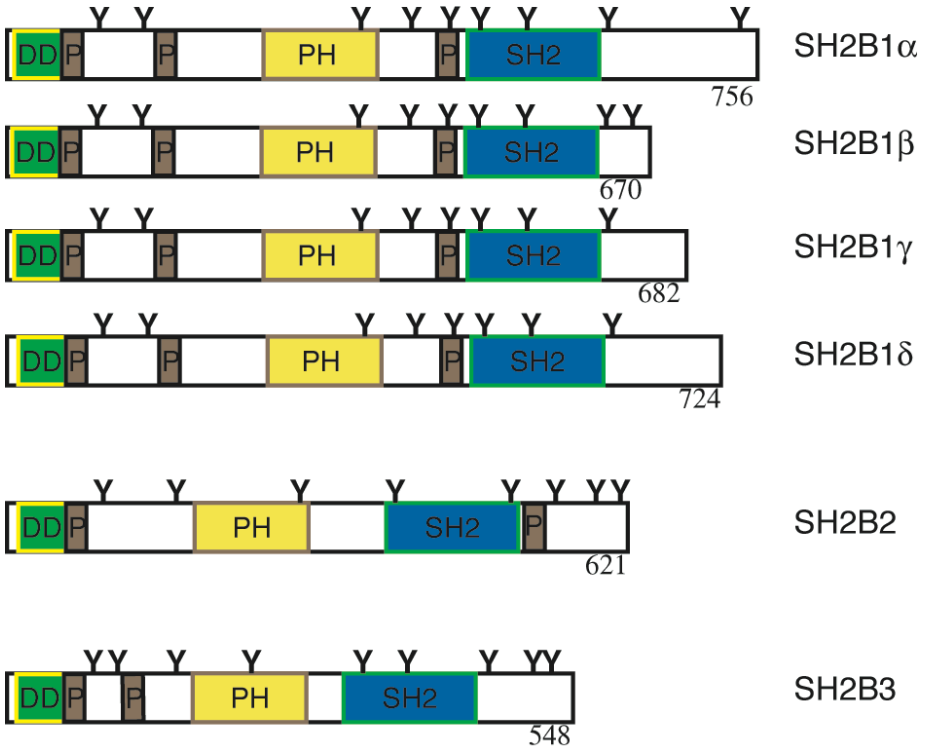


FIG. 1.1. Schematic of SH2B family members.
Schematics are shown for rat SH2B1 (isoforms α, β, γ, and δ), SH2B2, and SH2B3, with the numbers representing amino acids. The abbreviations used are DD for dimerization domain, P for proline-rich region, PH for pleckstrin homology domain, SH2 for Src homology 2 domain, and Y for tyrosine. Alternative splicing of SH2B1 results in 4 isoforms. Unique sequences for each isoform have been denoted in the schematic by the C-terminal shaded boxes labeled with the respective isoform letter, the orange boxes present in isoforms SH2B1γ and SH2B1δ indicate a shared sequence within the splicing region.

splicing at the 3' end, giving rise to four protein products, SH2B1α, β, γ, and δ (43) (41) (37) (46) (FIG 1.1). All four SH2B1 isoforms are identical with respect to their N-termini, including their PH and SH2 domains, and differ only at their C-termini shortly after the SH2 domain.

SH2B1 Associates with a Variety of Receptor and non-Receptor Tyrosine Kinases

As SH2B1 lacks intrinsic catalytic activity, a good deal of effort in the last decade has gone into establishing an adapter function for SH2B1 by identifying binding partners of SH2B1. TrkA, a variety of other receptor tyrosine kinases, and JAK2 a receptor-associated tyrosine kinase, were among the first characterized binding partners of SH2B1; many of these initial interactions were identified in yeast two-hybrid screens (34, 36-38, 40, 41). In order to fully understand SH2B1's function, it is important to understand the similarities and/or differences by which SH2B1 operates within the context of different tyrosine kinases.

Initial studies aimed at characterizing the interaction of SH2B1 with its upstream binding partners, were carried out in the context of GH activated JAK2. GH binds to the GH receptor a member of the cytokine family of receptors, and activates the GH receptor associated JAK2. Studies using 3T3-F442A preadipocyte cells demonstrated that SH2B1 selectively associates with endogenous GH-activated JAK2 (41). Glutathione-S-transferase pulldown, co-immunoprecipitation, and/or yeast two-hybrid assays using SH2B1α, β, and/or γ confirmed that maximal interaction of SH2B1 with JAK2 requires kinase-active, tyrosyl phosphorylated JAK2 and the SH2 domain of SH2B1 (41, 47). The primary JAK2 binding site for the SH2 domain of SH2B1 has been identified as phosphoTyr813 (48), which resides in a YXXL motif in the pseudokinase domain (JH2) of JAK2 adjacent to the region linking JH2 to the JH1 kinase domain. The JH2 domain has been hypothesized to be an autoinhibitory domain that gets displaced upon ligand binding to the associated cytokine receptor (49). This same phosphotyrosine (pTyr813) in JAK2 has been shown to bind the SH2

domain of another member of the SH2B family of proteins, SH2B2 (50), and an analogous tyrosine in JAK3 has been implicated as the binding site in JAK3 for the SH2 domain of SH2B1 (48). Although SH2B1 also binds to JAK1(42), JAK1 does not possess a tyrosine corresponding to phosphoTyr813 in JAK2 and its site of interaction with SH2B1 remains unknown.

Interestingly, in contrast to its binding site in JAK2 and JAK3, the binding site of the SH2 domain of SH2B1 is thought to reside within the kinase domain for TrkA and TrkB (34, 51, 52), insulin receptor (37, 53-56), and GDNF receptor RET (57), and in the kinase insert domain of PDGF receptor (58). Crystallographic studies with the SH2 domains of SH2B1 and SH2B2 and their binding sites in JAK2 and the insulin receptor indicate that this difference in binding site location influences which family member binds preferentially. Thus, the SH2 domain of SH2B2, which dimerizes, shows a preference for the insulin receptor, whereas the SH2 domain of SH2B1, which does not dimerize, shows a preference for JAK2 (59). It seems reasonable to speculate that these differences in binding site location might also influence which tyrosines in SH2B1 (and SH2B2) are phosphorylated by the associated kinase, what proteins SH2B1 (and SH2B2) could recruit to the different receptor complexes, and/or the ability of any recruited proteins to interact with additional binding partners.

In addition to the SH2-dependent interaction of SH2B1 with phosphoTyr813 in JAK2, there appears to be one or more lower-affinity interactions involving amino acids in SH2B1 that reside outside of the SH2 domain that allow it to bind to inactive JAK2 (60). The suggestion was made that the interaction via this site(s) in SH2B1 with inactive JAK2 might serve to increase the local concentration of SH2B1 around JAK2, thereby facilitating binding of the SH2 domain to ligand-activated JAK2. This would result in a more rapid and robust cellular response to hormones and cytokines that activate JAK2. Alternatively or additionally, this interaction, which appears to be inhibitory in nature, might also help prevent abnormal activation of JAK2. Interestingly, Miquet et al. (61) reported finding, in a mouse model of GH excess in which JAK2

was not phosphorylated, increased levels of SH2B1, particularly in membranes. Based upon this correlative study, the authors suggested that in this model, SH2B1 could be a negative regulator of JAK2. However, an SH2 domain-independent association has not been reported for SH2B1 and TrkA.

Phosphorylation of SH2B1

Activation of TrkA by NGF not only promotes SH2B1 binding to TrkA but also promotes the tyrosyl phosphorylation of SH2B1 (and SH2B2) (34, 39). The tyrosyl phosphorylation of SH2B1 is also observed upon activation of the insulin receptor, PDGF receptor, and FGF receptor (33, 40, 46, 62). These sites of phosphorylation have not yet been determined, however, insulin receptor has been reported to phosphorylate the SH2B family member SH2B2 on a tyrosine in its C-terminus (Tyr618) (63). This phosphotyrosine has been reported to bind the E2-dependent ubiquitin ligase c-Cbl and facilitate ligand-stimulated tyrosyl phosphorylation of c-Cbl, association of Cbl with the adapter protein Crk, and translocation of the GLUT4 glucose transporter to the plasma membrane (63). SH2B2 recruitment of c-Cbl has also been implicated in the ubiquitylation of the insulin receptor (64). Tyr618 in SH2B2 has been indirectly implicated as a target of erythropoietin-activated JAK2, important for the recruitment of c-Cbl and subsequent inhibition of EPO-dependent activation of the signal transducer and activator of transcription (Stat) 5 (65). The α isoform of SH2B1 has a similar tyrosine in its C-terminus, raising the possibility that it too can recruit c-Cbl. This phosphotyrosine is present only in the α isoform of SH2B1, while a different tyrosine is unique to the β isoform. Thus, although expected to recruit many of the same proteins, the α and β isoforms may be able to recruit a subset of unique binding proteins. Phosphorylation of a different subset of the tyrosines in SH2B1 by different receptor tyrosine kinases could also increase the diversity of SH2B1-mediated responses by allowing for the recruitment of receptor-specific phosphotyrosine binding partners. Support for the latter idea is provided by the finding that PDGF stimulates binding of p85 to SH2B1 whereas insulin and IGF-1

do not (66). As an SH2 domain-containing subunit of phosphatidyl inositol 3 (PI3) kinase, p85 would be expected to bind to phosphorylated tyrosines. While it is unknown which sites within SH2B1 are phosphorylated by the aforementioned receptor tyrosine kinases, the activation of JAK2 by GH has been shown to phosphorylate SH2B1β tyrosines 439 and 494 (67). Both of these sites were identified by two-dimensional phosphopeptide mapping as the major JAK2 phosphorylation sites in SH2B1β. A search for other JAKs that phosphorylate SH2B1 revealed that JAK1, but not JAK3, also phosphorylates SH2B1β when expressed in 293T or COS7 cells, with mutation of Tyr439 substantially diminishing the degree of phosphorylation. In contrast to wild-type SH2B1β, SH2B1β lacking these two tyrosines fails to enhance GH-induced membrane ruffling, suggesting that when phosphorylated by JAK2 in response to GH, Tyr439 and/or Tyr494 in SH2B1β recruits a protein(s) that links SH2B1β to the actin cytoskeleton. It remains to be seen whether NGF-activated TrkA phosphorylates these same tyrosines or different tyrosines within SH2B1. This is a particularly interesting matter since it is possible that these phosphorylated tyrosines in SH2B1 function as docking sites for other signaling molecules.

In addition to the tyrosyl phosphorylation of SH2B1, NGF stimulation of TrkA results in the phosphorylation of SH2B1 on multiple serines and/or threonines (68). One potential site of serine phosphorylation has been identified, serine 96, which has been shown to be phosphorylated by a kinase that lies downstream of MEK, e.g. ERK (68). Whether or not this phosphoserine, or other phosphoserines and/or threonines, are capable of recruiting proteins to SH2B1-TrkA complexes has not been studied. A level of SH2B1 Ser/Thr phosphorylation similar to that seen with NGFhas been observed upon activation of JAK2 by GH (41) and of PDGF receptor (60).

SH2B1 Binding Partners

Several other binding partners have been identified that appear to bind to domains in SH2B1 other than the SH2 domain or phosphorylated tyrosines. One of these is Rac, a major actin-regulating protein involved in cell ruffling,

lamellipodia formation, and cell motility (69-71). GH both activates Rac and promotes membrane ruffling in a variety of cell types (72-74). Consistent with SH2B1 participating in GH-stimulated actin reorganization and cell motility, both ectopically expressed and endogenous SH2B1 localize to membrane ruffles and lamellipodia. Further, overexpression of SH2B1 enhances GH-induced membrane ruffling, pinocytosis and lamellipodia formation in 3T3-F442A preadipocytes whereas SH2B1β lacking a functional SH2 domain [SH2B1β(R555E)] inhibits GH-stimulation of these functions. Finally, the same region of SH2B1β (amino acids 85-106 containing a proline-rich domain) found to be required for GH-induced cell motility is also required for SH2B1β to co-immunoprecipitate with Rac (73). These data suggest that SH2B1β enhances GH induced cell motility at least in part by recruiting Rac to GH-activated JAK2/GH receptor/SH2B1 complexes at the plasma membrane. It seems likely that SH2B1 will be found to bind additional proteins that interact with or regulate the actin cytoskeleton, particularly since one or both of the JAK2 phosphorylation sites in SH2B1β is required for the stimulatory effect of SH2B1 on membrane ruffling (FIG. 1.2, A).

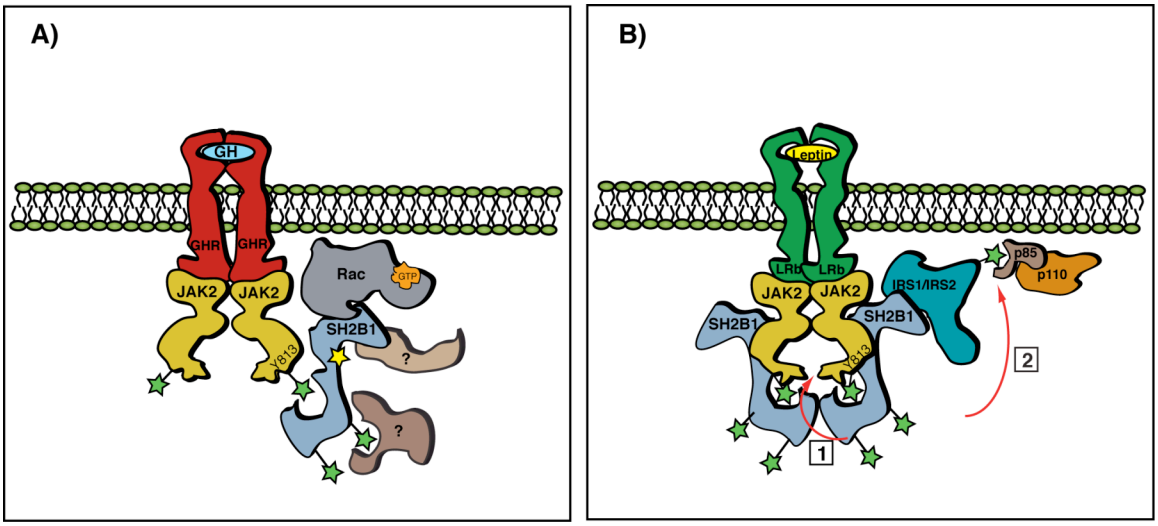


FIG. 1.2. The multiple roles for SH2B1.
(A), GH binding to the GH receptor leads to activation and autophosphorylation of JAK2. JAK2 tyrosyl phosphorylation (green stars) at amino acid 813 recruits the SH2 domain of SH2B1. JAK2 phosphorylates SH2B1β on tyrosines 439 and 494. These phosphotyrosines form potential binding sites for other signaling proteins. GH also promotes serine/threonine phosphorylation of SH2B1β (yellow stars). These phosphorylated amino acids as well as other protein-protein interaction domains in SH2-Bβ may serve to recruit proteins to GHR/JAK2/SH2B1β complexes, such as Rac (proteins with "?" represent potential SH2B1 interacting proteins). (B), Model of SH2B1 action in leptin signaling. SH2B1 binds to and potentiates activation of JAK2 in response to leptin, globally enhancing the activation of pathways downstream of JAK2 (box 1). SH2B1 binds simultaneously to both JAK2 and IRS1 or IRS2, mediating formation of a complex of JAK2, SH2B1, and IRS1 or IRS2. Consequently, SH2B1 mediates tyrosine phosphorylation of IRS1 and IRS2, resulting in activation of the PI3-kinase pathway (box 2).

A role for SH2B family members in the regulation of the actin cytoskeleton is further supported by the finding that SH2B2 also binds proteins involved in actin regulation. SH2B2 was reported to bind to Vav3, a guanine nucleotide exchange factor for Rac and other Rho family GTPases (75). Insulin was found to promote the binding of the N-terminal 182 amino acids of SH2B2 to Enigma and the co-localization of SH2B2 and Enigma in large F-actin containing ruffles (76). Enigma is a PDZ and LIM domain-containing protein that was previously shown to be associated with the actin cytoskeleton.

Interestingly, the overexpression of SH2B1 was found to enhance NGF-dependent neurite outgrowth of PC12 cells (33, 34). While the small GTPases Rac, Rho, and Cdc42 have each been implicated in regulating the growth cone activity of an elongating neurite (77, 78), the Rac binding site is surprisingly not required for SH2B1 to enhance NGF-mediated neurite outgrowth (79). This topic will be covered in greater detail in Chapter 4, however, these results suggest that GH and NGF-mediated changes to the actin cytoskeleton may employ very separate functions of SH2B1.

Additionally, SH2B1 has also been reported to recruit other adapter proteins. Qian et al. (34) found SH2B1 to co-immunoprecipitate with the small adapter protein Grb2, implicated in the regulation of ERKs 1 and 2. Ren et al. (80) found SH2B1 to recruit the much larger adapter insulin receptor substrate (IRS) adapter proteins to activated JAK2 in response to leptin. Leptin is a hormone secreted by adipose tissue that regulates energy homeostasis through activation of its long isoform receptor (LRb) in hypothalamic neurons (81). Leptin binding to LRb activates the LRb-associated JAK2, which in turn phosphorylates and activates downstream signaling proteins, including the IRS proteins (82-84) and the transcription factor Stat3 (85-88). Loss of either leptin or LRb in mice results in morbid obesity (89-92). In HEK cells stably expressing LRb, SH2B1β has been shown to recruit IRS1 and IRS2 to JAK2, and promote both the formation of a ternary complex containing JAK2, SH2B1 and either IRS1 or IRS2 (93) and the subsequent tyrosyl phosphorylation of IRS1 and IRS2 (93) (FIG. 1.2,

B). The phosphorylated tyrosines in IRS proteins bind the SH2 domain of the PI3 kinase regulatory subunit p85, the initial and rate limiting step in the activation of the PI3-kinase pathway (94). The PH domain of SH2B1 is required for the interaction of SH2B1 with IRS proteins (93). Interestingly, in addition to enabling leptin-dependent activation of PI3-kinase by recruiting IRS proteins, SH2B1 also has the potential to influence leptin-dependent activation of both PI3-kinase and Stat3 by a completely different mechanism, one that involves SH2B1 binding only to JAK2.

SH2B1 Enhancement of Tyrosine Kinase Activity

Although SH2B1 plays an adapter role in the recruitment of Rac, Grb2, IRS1, IRS2, and presumably other proteins to JAK2, its influence on JAK signaling pathways appears to extend beyond that of a classic adapter protein. I*n vitro* kinase assays and anti-phosphotyrosine immunoblots of JAK2 suggested that SH2B1 can directly stimulate JAK2 kinase activity and autophosphorylation (95) (47). SH2B1α and β were found to enhance GH-dependent tyrosyl phosphorylation of JAK2 and Stat5b by a mechanism requiring the SH2 domain of SH2B1 (95) (47) (FIG. 1.3). Enhancement by SH2B1 of JAK2 activity appears to be unique within the JAK family because overexpression of SH2B1β does not similarly enhance the autophosphorylation of JAK1 or JAK3 (42). The latter was somewhat surprising because SH2B1 binds to JAK3 at a site analogous to its binding site in JAK2 (48).

Two mechanisms have been proposed for how SH2B1 enhances JAK2 activity. The first is that dimerization of SH2B1 leads to dimerization of its associated JAKs, which in turn enhances JAK2 activity (47).

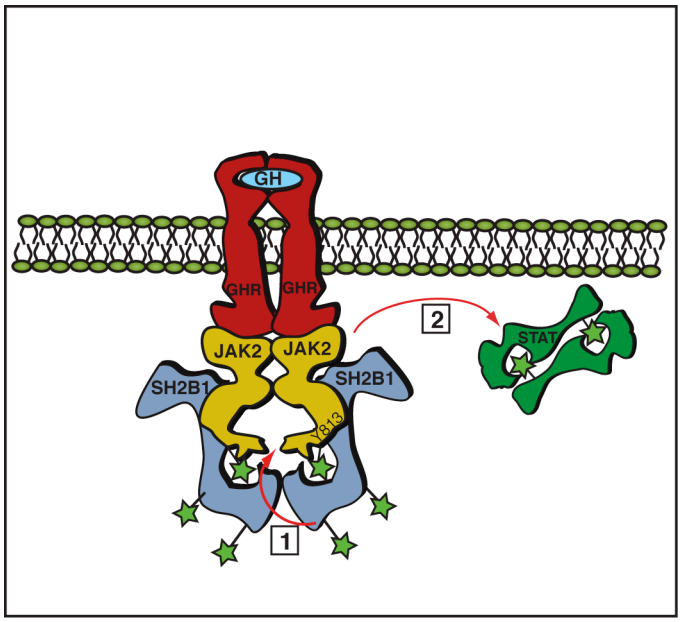


FIG. 1.3. SH2B1 enhances JAK2 kinase activity.
SH2B1 directly enhances JAK2's kinase activity (box 1) resulting in an increase in phosphorylated STAT5b (box 2) and presumably other substrates of JAK2.

In support of this, an N-terminal dimerization domain (amino acids 24-85 of rat SH2B1) was identified and found to be required for SH2B1 to induce dimerization of JAK2 in a yeast two-hybrid assay. Furthermore, SH2B1 lacking that domain acts as a dominant negative for GH-induced activation of JAK2. An alternative model suggested by Kurzer et al. (50) is that SH2B1 increases the number of active JAK2 molecules by stabilizing a conformational change in JAK2 that maintains JAK2 in an active state. This model is based in part upon two main findings. The first was that, in an overexpression system, the SH2 domain of SH2B1, which binds only to the phosphorylated form of Tyr813, is sufficient to activate JAK2. The second is that the SH2B1-mediated increase in JAK2 dimerization occurs even if only one SH2B1 can bind to the dimer. This model is based on the idea that JAK2 must first be active (and autophosphorylated) to bind SH2B1 at phosphoTyr813 and that the binding of SH2B1 causes a conformational change that maintains JAK2 in an active state. Dimerization of SH2B1 is hypothesized to enhance the binding of SH2B1 to an already existing JAK2 dimer. Importantly, both models agree that the ability of SH2B1 to enhance JAK2 activity is a direct function of SH2B1, and not a result of competition with a JAK2 inhibitor or recruitment of an activator of JAK2.

The ability of SH2B1 to enhance tyrosine kinase activity appears not to be unique to JAK2. In addition to JAK2, SH2B1 has been reported to positively regulate the kinase activity of TrkA (96), FGF receptor (40), and insulin receptor (62, 93, 97). The ability of SH2B1 to enhance the kinase activity of TrkA will be discussed more thoroughly later in this Chapter.

Phenotype of SH2B1$^{-/-}$ Mice

The generation of SH2B1$^{-/-}$ mice has revealed a number of physiological roles for SH2B1 consistent with its role as an adapter and/or enhancer protein for JAK2. However, there is only limited published work from these SH2B1$^{-/-}$ mice regarding the role of SH2B1 in TrkA function. One of the most notable phenotypes consistent with SH2B1 acting in JAK2 signaling pathways is the

severe hyperphagia and obesity observed by Ren et al. (80) in SH2B1$^{-/-}$ mice. SH2B1$^{-/-}$ mice grew to twice the body weight of wild-type littermates within 21 weeks from birth. In accordance with the ability of SH2B1 to enhance leptin action, the obesity in the SH2B1$^{-/-}$ mice was accompanied by severe leptin resistance as characterized by substantially elevated plasma leptin levels. Plasma leptin levels were found to be dramatically elevated in these mice prior to the onset of their obesity, consistent with hyperleptinemia being a cause rather than a corollary of obesity in these mice. A second group of investigators reported that after 6 to 10 weeks, the size and weight of their SH2B1$^{-/-}$ mice were similar to that of wild-type mice (98). The discrepancy between the two sets of findings has been hypothesized to potentially result from differences in diet and level of environmental stress (80). Both groups did, however, report an initial postnatal growth retardation and proportionate dwarfism, consistent with a stimulatory effect of SH2B1 on GH action. However, Ohtsuka et al. (98) did not find evidence for the enhancer effect of SH2B1, i.e. enhanced activation of JAK2. They did not observe a reduced stimulatory effect of the JAK2 activator, interleukin 3, on responses of MAST cells from SH2B1$^{-/-}$ mice. They also reported finding no difference in the ability of GH to stimulate Stat5 activation in the liver. Whether this lack of an expected decreased response in MAST cells and liver from SH2B1$^{-/-}$ mice is because the enhancer effect of SH2B1 seen in cell culture and *in vitro* does not manifest itself in the context of the whole animal, the experiments were not done in such a way to observe the enhancer effect of SH2B1, or whether the absence of SH2B1 was compensated for during development is not known.

The SH2B1$^{-/-}$ mice exhibited additional phenotypes that are consistent with SH2B1 acting as a positive regular for IGF-1 and insulin. Both groups reported that SH2B1$^{-/-}$ mice had impaired fertility with Ohtsuka et al. (98) noting that the developmental defects in gonadal organs were similar to those seen in mice with IGF-I or follicle-stimulating hormone (FSH) receptor deficiencies. These results are consistent with SH21β playing a positive role in the IGF-I pathway that up-

regulates levels of FSH receptor. Although Ohtsuka et al. (98) mentioned seeing no differences in blood glucose levels, Duan et al. (99) observed age-dependent hyperinsulinemia, hyperglycemia and glucose intolerance in the SH2B1$^{-/-}$ mice. Consistent with SH2B1$^{-/-}$ mice exhibiting insulin insensitivity, Duan et al. also observed impaired insulin receptor activation and signaling in the liver, skeletal muscle and fat, including impaired tyrosine phosphorylation of IRS1 and 2 and activation of PI3 kinase kinase/Akt and Erk1/2 pathways. Examination of pancreatic islets in SH2B1$^{-/-}$ mice revealed enlarged islet size in mice, but no abnormalities of the insulin-producing β cells, suggesting that SH2B1 is not required for β cell growth and function. These findings complement the data obtained from immortalized cell lines, and suggest a role for SH2B1 in maintaining insulin/glucose balance. Whether SH2B1$^{-/-}$ mice exhibit other phenotypes predicted from the role of SH2B1 as a signaling protein for TrkA has not been rigorously examined. Ohtsuka et al. (98) reported that sensory neurons from SH2B1$^{-/-}$ mice were able to undergo neurite outgrowth in response to NGF, although they did not provide the data concerning this particular observation. Unfortunately, neither study has provided characterization of the sympathetic nervous system of the SH2B1$^{-/-}$ mice.

Interestingly, by using a neuronal specific enolase promoter to drive transgenic expression of SH2B1β in the systemic SH2B1 knockout mice (SH2B1^{TgKO}), Ren et al. (100) were recently able to rescue the obesity and hyperlipidemia observed in the SH2B1^{KO} mice. The characteristic leptin and insulin insensitivity of the SH2B1^{KO} mice was similarly rescued in the SH2B1^{TgKO} mice, suggesting that restoring the levels of neuronal SH2B1β alone is sufficient to restore leptin and insulin action (100). As mentioned earlier, the systemic deletion of SH2B1 significantly reduced leptin signaling, resulting in impaired leptin-induced activation of hypothalamic JAK2 (80). The study supports a conclusion in which both the enhancer and adapter functions of SH2B1β are required for proper leptin signaling. As an enhancer of JAK2, SH2B1β amplifies JAK2 phosphorylation and activation of Stat3 (80) (100). As an adapter, SH2B1β

also facilitates the second arm of the leptin signaling cascade in which SH2B1β recruits IRS2 to JAK2 resulting in the activation of the PI3K pathway, required for the regulation of energy metabolism (101). While the possibility remains that restoration of neuronal SH2B1β regulates obesity by additional leptin-independent mechanisms, the study underscored the importance of SH2B1 for neuronal function.

Evidence for the Role of SH2B1β in Neuronal Differentiation and Survival

As mentioned previously, SH2B1 was implicated in neuronal function several years ago (33, 34). Qian et al. (34) identified both SH2B1 (recently identified as the γ isoform, Doche, M., Maures, T., J., and Carter-Su, C., unpublished observation) and SH2B2 as TrkA, B, and C binding proteins, using a yeast two-hybrid assay employing a cDNA library isolated from rat spinal cord and dorsal root ganglion. Attempts to determine exactly which tyrosine residue within TrkA mediated the interaction with SH2B1 were hampered because three of the tyrosines (Y679, Y683, and Y684) within the kinase activation loop TrkA, are required for catalytic activity (34). Using a yeast two-hybrid assay, Qian et al. (34) demonstrated that SH2B1γ bound to a mutant TrkA in which all but the three tyrosines within the activation loop were mutated to phenylalanines. Rui et al. (33) confirmed the phosphotyrosine-dependent interaction between SH2B1β and TrkA by demonstrating that GST-SH2B1β pulled down TrkA only from lysates of NGF-treated PC12 cells. Additionally, the GST-SH2B1β(R555E) mutant, which contains a functionally inactivating point mutation within its SH2 domain, was unable to pull down TrkA from cells with or without prior NGF treatment, indicating that SH2B1 requires a functional SH2 domain for its interaction with TrkA

The rapid NGF-dependent association of SH2B1 to TrkA mirrored in timing the NGF-dependent association of well known TrkA effector molecules Shc and PLC-γ. Upon NGF stimulation, Shc and PLC-γ are recruited to phospho-tyrosine residues 499 and 785, respectively, where they are then tyrosyl phosphorylated by active TrkA (102) (103, 104). Similarly, the NGF-dependent association between SH2B1 and TrkA resulted in the rapid tyrosyl phosphorylation of SH2B1 in neonatal sympathetic neurons (34) as well as in PC12 cells (33). While TrkA-

dependent phosphorylation of PLC-γ triggers its activation (105), SH2B1 contains no intrinsic enzymatic activity. Therefore, the SH2B1 association with TrkA more closely resembles that of the adapter protein Shc, which links TrkA receptors to Grb2, SOS, and the Ras-ERK signaling cascade (28, 104). Upon NGF activation of TrkA, Grb2 is recruited to the receptor complex through the association between its SH2 domain and a phosphotyrosine docking site on Shc (106). Myc-tagged SH2B1γ was also shown to immunoprecipitate Grb2 in 293 cells (34). However, the SH2B1γ/Grb2 interaction seen in 293 cells co-expressing TrkA and SH2B1 was dependent upon a proline rich region in SH2B1 and was observed whether or not cells had been treated with NGF (34).

The TrkA/SH2B1 interaction was hypothesized to play a central role in the specific activation of Ras-dependent or Ras-independent signaling cascades (34). Indeed, dissociated neonatal sympathetic neurons cultured in the presence of NGF exhibit potently reduced the survival ratse when α-SH2B1 antibodies were introduced by trituration (34). In addition to promoting the survival of neurons, SH2B1γ demonstrated an ability to regulate the maintenance of axonal processes of neurons within explants of sympathetic ganglia. Sympathetic neurons were transfected with cDNA encoding either SH2B1γ or a truncation mutant of SH2B1γ containing only the C-terminal portion (including the full SH2 domain). In the presence of NGF, neurons overexpressing SH2B1γ displayed long and branched axonal processes, while neurons overexpressing the truncation mutant of SH2B1γ exhibited significant axonal degeneration after 2 days (34). Similarly, overexpression of SH2B1γ (34) and SH2B1β (33) enhanced NGF-dependent neurite outgrowth of PC12 cells. Taken together, these results implicated SH2B1 as an important mediator of Trk receptor signaling, that contributes to both neuronal differentiation and survival.

However, the precise mechanism by which SH2B1 mediates these effects has yet to be determined. Studies performed by the Ginty laboratory (34, 96) favor a mechanism whereby SH2B1 performs an adapter/enhancer function for

Trk receptors, much like the mechanism described above for leptin signaling. Specifically, when co-expressed with ERK1 and TrkA in 293 cells, SH2B1γ enhanced the NGF stimulation of ERK1. The association of SH2B1γ with both Grb2 and active TrkA was required for enhancement of ERK1 phosphorylation (34). Additionally, overexpression of SH2B1γ was found to potentiate the activity of TrkA (96). PC12 cells stably overexpressing full-length SH2B1γ enhanced both the magnitude and duration of NGF-induced TrkA phosphorylation at tyrosine 490 (Shc binding site in rat) as well as the phosphorylation of ERKs 1 and 2 (96). This was assessed by stimulating the cells with a pulse of NGF (100 ng/ml) for 10 minutes before replacing with new medium containing a function blocking NGF antibody. However, PC12 cells stably overexpressing an N-terminal truncation mutant of SH2B1γ(ΔN237) were unable to potentiate NGF-induced activity of TrkA and also displayed reduced phosphorylation of ERKs 1 and 2 compared to control cells (96). The importance of the N-terminal portion of SH2B1γ was thought to arise from a multimerization domain located between amino acids 100-237 (96). This multimerization domain was proposed to facilitate the formation of homo or heteropentamers of SH2B1 and SH2B2, according to the results of size exclusion chromatography from 293 cells overexpressing SH2B1 eluted as part of a ~440 kDa complex (96). While multimerization of SH2B1 was found to be independent of NGF stimulation, Qian et al. (96) concluded that multimerization was required to enhance NGF-dependent TrkA activity.

The Ginty group also examined whether multimerzation of SH2B1 is required for the enhancement of NGF-dependent neurite outgrowth (96). Using a PC12 cell line that expresses little to no TrkA (PC12nnr5) and is unresponsive to NGF, Qian et al. (96) transiently expressed mutant TrkA lacking all conserved tyrosines except for those within the activation loop, as well as wild-type SH2B1γ, SH2B1γ(ΔN237), or vector control. Only cells expressing full-length SH2B1γ were able to facilitate NGF-dependent neurite formation (96). These results

suggest that the N-terminal 237 amino acids are required for SH2B1's role in NGF-dependent neurite outgrowth (96).

Collectively, these results suggested to Qian and Ginty (96) that the formation of pentameric complexes of SH2B1γ potentiate the NGF-induced activity of TrkA. SH2B1γ also functions to couple Grb2 to active TrkA, thereby transducing the signal through a Ras-dependent mechanism (34). Enhancement of NGF-mediated TrkA autophosphorylation by SH2B1γ thus leads to an enhanced and prolonged activation of ERKs 1 and 2, which drives neuronal differentiation (96).

The mechanism put forth by Qian and Ginty, however, contains several points of contention within the field. Rui et al. (33, 68) similarly demonstrated that NGF promotes a rapid association of SH2B1 with TrkA and subsequent tyrosyl phosphorylation of SH2B1 on tyrosines as well as serines/threonines. Furthermore, the overexpression of SH2B1β within PC12 cells significantly enhanced NGF-dependent neurite outgrowth while overexpression of SH2B1β(R555E), which cannot bind to TrkA, inhibited NGF-dependent differentiation in a dominant negative manner (33). While the aforementioned results are all consistent with the mechanisms proposed by Qian et al. (34, 96), a critical incongruity between the groups is whether SH2B1 actually enhances the activity of TrkA in response to NGF. Even though overexpression of SH2B1β and SH2B1β(R555E) demonstrate opposing effects with regard to NGF-dependent neurite outgrowth, neither augmented the NGF-induced autophosphorylation of TrkA compared to GFP-control, assessed using a general anti-phosphotyrosine antibody (33). Moreover, NGF-induced phosphorylation of Shc, PLC-γ, or ERKs 1 and 2 was also not substantially affected by overexpression of SH2B1β or SH2B1β(R555E) (33). These findings indicated that while overexpression of SH2B1β may cause indirect enhancement of TrkA or ERKs 1 and 2, the dominant negative effect of overexpressed SH2B1β(R555E) on NGF-dependent neurite outgrowth is unlikely to be a result of reduced TrkA

activation or a diminished ability of TrkA to phosphorylate the substrates responsible for the Shc/Ras/MEK/ERK cascade or PLCγ pathways (33). These results suggest that SH2B1β might facilitate NGF-dependent differentiation through a novel pathway at a point downstream of or parallel to ERKs 1 and 2. Although both SH2B1γ and SH2B1β facilitate a similar level of enhancement of NGF-dependent neurite outgrowth (33, 34), it is possible that the use of different isoforms of SH2B1 could explain the observed discrepancy. A direct comparison of each isoform's ability to modulate NGF-induced TrkA activity has yet to be made. In any case, there remains an unexplained mechanism by which the β isoform of SH2B1 enhances NGF-dependent neurite outgrowth.

The second discrepancy concerning the findings of Qian et al. (96) pertains to the multimerization domain. As mentioned previously, a ~440 kDa complex containing SH2B1 was obtained using a size exclusion chromatography column (34). Because SH2B1γ is approximately 87 kDa, the conclusion was that SH2B1α assumes a pentameric conformation in cells, mediated by a multimerization domain (a.a. 100-237). Qian et al. (96) unambiguously demonstrated that SH2B1 is at least capable of forming dimers by co-immunoprecipitating differentially tagged forms of SH2B1. However, the study failed to provide sufficient evidence that the 440 kDa complex contained SH2B1 only and not endogenous interacting partners of SH2B1. Adding to the uncertainty of this finding, Nishi et al. (47) identified a dimerization domain within SH2B1 (amino acids 26-84) and SH2B2 (23-81) that is both necessary and sufficient to mediate dimerization of SH2B1. Based upon X-ray crystallography of SH2B2, a molecular model of SH2B1 revealed a novel dimerization motif in which a stretch of phenylalanines of the two monomers interdigitate, analogous to the previously identified leucine zipper (47). In contrast to the multimerization domain reported by Qian et al.(96), the phenylalanine zipper is only capable of facilitating the formation of SH2B1 homo- or heterodimers with SH2B2. These results do not support the existence of SH2B1 pentamers, but support the finding that the N-terminal portion of SH2B1 is responsible for dimerization and

potentially, the activation of (receptor) tyrosine kinases (34, 47). Unfortunately, an SH2B1γ N-terminal truncation mutant lacking only the first 100 amino acids and thus the phenylalanine zipper (47), was tested only for its ability to multimerize. The NΔ100 truncation mutant was not tested for its ability to activate TrkA or its ability to enhance NGF-induced neurite outgrowth. Therefore, the conclusion that SH2B1γ requires the N-terminus for enhancing the activity of TrkA cannot be substantiated. The only N-terminal truncation mutant thoroughly tested was SH2B1α(NΔ237) (96). As mentioned previously, overexpression of this mutant failed to enhance, but did not inhibit, TrkA activation or NGF-dependent differentiation of PC12 cells (96). However, the NΔ237 truncation removed several critical domains from SH2B1γ, including a functional nuclear export sequence (NES), as described in (79). Because the NΔ237 truncation both disrupts the subcellular localization of SH2B1 and likely compromises its nuclear function, it is difficult to distinguish the reason SH2B1α(NΔ237) failed to enhance NGF-dependent differentiation.

A Nuclear Role for SH2B1

A common assumption in all of the aforementioned studies of SH2B1 is that SH2B1 localizes and functions at the plasma membrane. Its presence at the plasma membrane and its interactions with numerous activated JAK and receptor tyrosine kinases normally found at the plasma membrane indicate that SH2B1 does indeed carry out functions at the plasma membrane. However, while investigating the ability of SH2B1β to enhance NGF-induced neuronal differentiation of cultured PC12 cells, Chen et al. (79) made the unexpected finding that SH2B1β undergoes constitutive nucleocytoplasmic shuttling, even though at steady state, it appears to resides primarily at the plasma membrane and in the cytoplasm. Inhibition of Crm1-mediated nuclear export with leptomycin B caused both endogenous and ectopically expressed SH2B1β to accumulate in the nucleus. Nuclear accumulation of SH2B1β was also seen after deleting or mutating a previously unrecognized nuclear-export sequence (NES). Importantly,

deletion or mutation of the NES (79) within SH2B1β destroys the ability of overexpressed SH2B1β to enhance NGF-dependent differentiation of PC12 cells but not the ability of SH2B1β to bind TrkA. The presence of the NES in all isoforms of SH2B1 suggests that all isoforms of SH2B1 will be found to undergo nucleocytoplasmic shuttling. Together, these findings suggest that SH2B1 must undergo nucleocytoplasmic shuttling for it to enhance NGF-induced neuronal differentiation.

Thesis Summary

Clearly, SH2B1 plays an important role in the signal transduction for several receptor and cytoplasmic tyrosine kinases. As suggested by the evidence presented thus far, SH2B1 is a unique molecule capable of functioning as both an adapter as well (or simultaneously) as an enhancer of tyrosine kinase activity. However, the mechanism by which SH2B1 enhances neuronal differentiation and survival in response to NGF is not clear. In contrast to findings from the Ginty laboratory (34), Rui et al. (33) concluded that SH2B1β mediated NGF's effects in a manner independent of the Ras/MEK/ERK or PLCγ pathways, possibly through a novel pathway. Therefore, the aim of this thesis project was to elucidate the mechanism by which SH2B1β enhances NGF-dependent differentiation and survival of neurons.

In Chapter 2, we first sought to determine the mechanism by which SH2B1β enhances NGF-dependent survival of neurons. In both primary sympathetic neurons and terminally differentiated PC12 cells, the removal of neurotrophic factors results in the retraction and degeneration of neurite outgrowths and eventual apoptosis (107, 108). NGF is a potent survival factor, and protects PC12 cells from apoptosis induced by neurotrophic factor withdrawal (108, 109), cytotoxic drugs (110-112), and oxidative stress (113-115). The anti-apoptotic properties of NGF are elicited through the activation of the PI3K/Akt pathway (116). Thus, we hypothesized that SH2B1β may enhance the NGF-induced activation of Akt.

Indeed, as reported in Chapter 2, overexpression of GFP-SH2B1β in PC12 cells was found to enhance NGF-dependent activation of Akt kinase activity as well as the phosphorylation of downstream Akt targets GSK-3α/β, FKHRL1 (FoxO3a), and FKHR (FoxO1). However, the NGF-dependent activation of Akt

and the phosphorylation of the downstream effectors were found not to be inhibited by overexpression of SH2B1β(R555E), which functions as a dominant negative. Normal NGF-induction of the Akt pathway in cells overexpressing SH2B1β(R555E) was especially surprising in light of our unpublished observations that SH2B1β(R555E) promotes PC12 cell death and the finding from Qian et al. (34) that expression of the N-terminal truncation of SH2B1 leads to the degeneration of sympathetic neurons. Interestingly, we found that overexpression of SH2B1β(R555E) inhibits the redistribution of FoxO1 from the nucleus to the cytoplasm. FoxO1 functions as a pro-apoptotic transcription factor (117-119) as well as a negative regulator of differentiation (120). Direct phosphorylation of FoxO1 by Akt is known to be required for the formation of a 14-3-3/FoxO1 complex, which is then exported from the nucleus and tethered within the cytoplasm (118). As mentioned, NGF-induced FoxO1 phosphorylation was normal in SH2B1β(R555E) expressing cells, although FoxO1 remained nuclear. These results suggested that SH2B1β might directly participate in the regulation of the nuclear export of FoxO1 in addition to enhancing the Akt pathway.

Concurrent with the aforementioned finding, Dr. Linyi Chen in the Carter-Su laboratory discovered that SH2B1β contains a nuclear export sequence and undergoes nucleocytoplasmic shuttling. The nuclear presence of SH2B1β was especially surprising since SH2B1β was thought to localize and function only at the plasma membrane (39). Interestingly, blocking the nuclear export of SH2B1β also blocked SH2B1β enhancement of NGF-dependent differentiation in PC12 cells. Together, Dr. Linyi Chen and I hypothesized that SH2B1β's newfound nuclear localization may account for the ability of SH2B1β to enhance NGF-dependent neuronal differentiation and survival.

In Chapter 3, collaborative work between Dr. Chen and I demonstrated that SH2B1β enhances expression of a subset of NGF-sensitive genes important for differentiation. Using microarray analysis of PC12 cells, we established that

the overexpression of SH2B1β leads to altered transcription of only a specific subset of NGF-responsive genes. If SH2B1β enhances NGF-induced neuronal differentiation of PC12 cells primarily by enhancing NGF-activation of TrkA as has been hypothesized (96), we would expect a corresponding general enhancement of NGF induction or inhibition of NGF-responsive gene expression. Instead, of the 511 genes whose expression at least doubled after NGF treatment in control cells, only 34 demonstrated a further NGF-dependent doubling in SH2B1β overexpressing PC12 cells. Among the subset of NGF-responsive genes that were enhanced by the presence of SH2B1β, we identified Plaur (uPAR), Mmp3, and Mmp10 whose gene products encode urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 3 (MMP3), and matrix metalloproteinase 10 (MMP10). These proteins were of particular interest because uPAR has been shown to be required for NGF-mediated neuronal differentiation (121, 122) and MMP3, present in growth cones of NGF-treated PC12 cells, has been implicated in neurite penetration through the extracellular matrix (123). Moreover, uPAR, MMP 3, and MMP 10 are all situated in the same proteolytic cascade responsible for extracellular matrix degradation required for neurite penetration through the extracellular matrix. Interestingly, the PC12 cells expressing the dominant negative SH2B1β(R555E) are not only unable to undergo NGF-dependent neurite outgrowth, but they also demonstrate a coincident inability to promote NGF-induced transcription of Plaur, Mmp3, and Mmp10 (124). These results suggest that SH2B1β may mediate NGF-induced neuronal differentiation and neurite outgrowth at least in part by enhancing NGF-dependent expression of uPAR, MMP3, and MMP10. Accordingly, depletion of endogenous SH2B1 using RNA interference blocked the ability of NGF to induce transcription of these genes. In light of these findings, we hypothesized that the nucleocytoplasmic shuttling by SH2B1β is required for SH2B1β enhancement of NGF-induced neuronal differentiation and is of consequence to the ability of SH2B1β to facilitate transcription of the genes encoding uPAR, MMP3, and MMP10. In Chapter 4, I wanted to directly address this hypothesis by identifying

a nuclear localization sequence (NLS) within SH2B1β. I found that disrupting SH2B1β translocation into the nucleus abolished the ability of SH2B1β to enhance NGF-induced neuronal differentiation of PC12 cells. Surprisingly, both the SH2B1β nuclear import mutant [SH2B1β(mNLS)] as well as the nuclear export mutant [SH2B1β(ΔNES)] blocked the ability of SH2B1β to enhance NGF-induced transcription of Plaur, Mmp3, and Mmp10. The inability of the nuclear localization defective SH2B1β(mNLS) to enhance NGF-induced differentiation or gene expression appears not to be a consequence of SH2B1β(mNLS) compromising the NGF signaling cascade because the extent and duration of phosphorylation of NGF effector molecules were unaffected in the SH2B1β(mNLS) expressing cells compared to SH2B1β expressing cells. Together these findings suggest that SH2B1β plays a critical role in NGF-mediated neuronal differentiation by facilitating enhancement of NGF-mediated gene expression through a novel nuclear mechanism.

In order to shed light on SH2B1β's role in the nucleus I wanted to establish nuclear binding partners for SH2B1β with the expectation that they may provide a functional context for SH2B1β. Therefore, I performed a tandem affinity purification (TAP) of SH2B1β using cytoplasmic/nuclear lysates from NGF-treated PC12 cells. The purification and subsequent MS/MS yielded several potential binding proteins. In Chapter 5, I identfied cell division cycle associated-7 (CDCA7) as a novel nuclear binding partner for SH2B1β. Described as a putative transcription factor, overexpression of CDCA7 in Rat1a cells induced anchorage-independent growth (125). In my hands, GFP-tagged CDCA7 dramatically blocks NGF-dependent differentiation, however, preliminary data suggest that the inhibition can be rescued by co-expression with SH2B1β. The association between CDCA7 and SH2B1 was confirmed by an NGF-dependent co-immunoprecipitation of overexpressed CDCA7 with endogenous SH2B1. However, the co-immunoprecipitated SH2B1 has a molecular weight of 110 kDa, and thus co-migrates with a specific highly phosphorylated form of SH2B1β or

possibly the δ SH2B1 isoform. We therefore tested the ability of SH2B1δ to bind. Upon expression of SH2B1δ, we observed surprising steady state localization at the plasma membrane, nucleoplasm, and nucleolus. Overexpressed CDCA7 was able to co-immunoprecipitate overexpressed SH2B1δ in an NGF-dependent manner. Collectively, these findings suggest that SH2B1β may relieve the actions of a pro-mitogenic transcription factor, allowing the cells to undergo neurogenic changes in response to NGF (FIG 1.4).

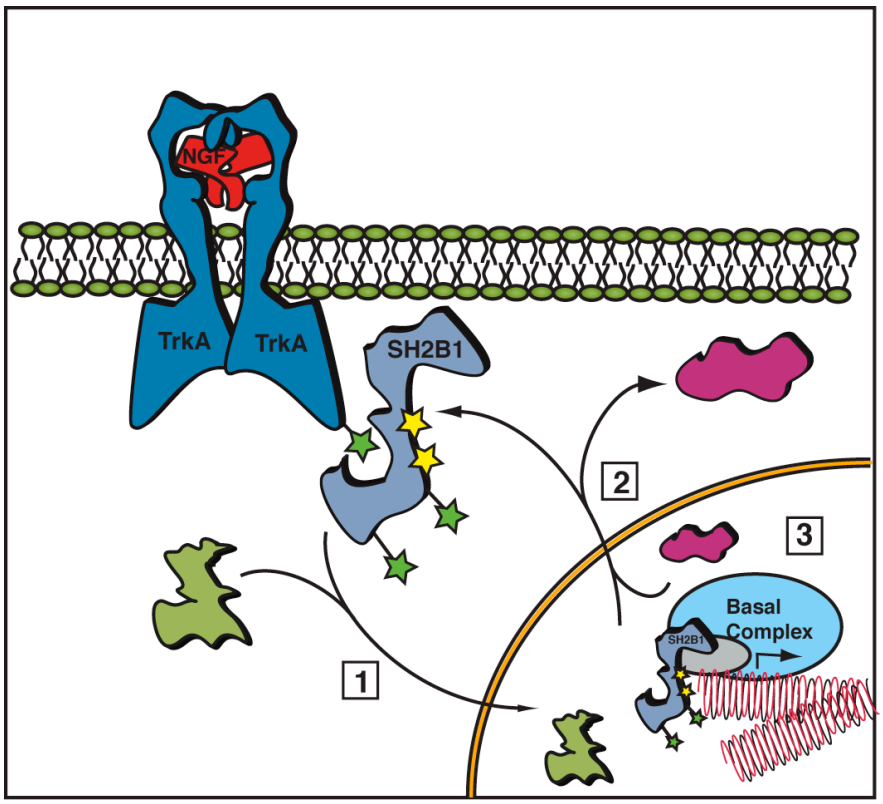


FIG. 1.4. A nuclear role for SH2B1.
Binding of NGF to TrkA causes activation and autophosphorylation of TrkA. SH2B1β is tyrosyl phosphorylated (green stars) by TrkA and serine/threonine phosphorylated (yellow stars) by downstream kinases. Hypothetical roles for nuclear SH2B1β include shuttling activators (box 1) or repressors (box 2) of NGF dependent transcription in or out of the nucleus, respectively. SH2B1β may also directly enhance transcription of NGF-responsive genes, perhaps as serving as an adapter protein for regulators of transcription. (3).

Portions of Chapter 1 are found in my review of SH2B1 published in TRENDS in Endocrinology and Metabolism (2007). Vol 18, 1, under the title "*SH2B1 and JAK2 a multifunctional adapter protein and kinase made for each other*" by Travis J. Maures, Jason H. Kurzer, and Christin Carter-Su.

Chapter 2

SH2B1 Is a Positive Regulator of Nerve Growth Factor-mediated Activation of the Akt/Forkhead Pathway in PC12 Cells

Abstract

To gain insight into the mechanism by which the adapter protein SH2-B promotes nerve growth factor (NGF) mediated neuronal differentiation and survival, the effect of SH2-B on the serine/threonine kinase Akt/PKB and downstream effector proteins was examined. PC12 cells stably overexpressing SH2-Bβ, which exhibit enhanced NGF-induced neuronal differentiation compared to control cells, showed enhanced and prolonged NGF-induced phosphorylation of Akt on Ser473 and Akt enzymatic activity. Surprisingly, NGF-induced phosphorylation of Akt on Ser473 and Akt activity were not altered in cells overexpressing SH2-Bβ(R555E) with a defective SH2 domain, despite the ability of the overexpressed SH2-Bβ(R555E) to block NGF-induced differentiation. Consistent with SH2-Bβ enhancing the activity of Akt, cells overexpressing SH2-Bβ but not SH2-Bβ(R555E) exhibited increased and/or prolonged phosphorylation of the pro-apoptotic Akt effector proteins, glycogen synthase kinase-3 (GSK-3), and Forkhead transcription factors, FKHRL1/FOXO3 and FKHR/FOXO1. Immunolocalization studies indicated that while ectopically expressed FKHR was primarily concentrated in the cytoplasm of control cells and cells transiently overexpressing SH2-Bβ, it was concentrated in the nucleus of cells transiently overexpressing SH2-Bβ(R555E). Similarly, SH2-Bβ stimulated the accumulation of FKHR in the cytoplasm of 293T and Cos-7 cells, whereas SH2-Bβ(R555E) enhanced its accumulation in the nucleus. In PC12 cells stably expressing forms

of SH2-Bβ, SH2-Bβ mimicked the ability of NGF to promote redistribution of FKHR to the cytoplasm whereas SH2-Bβ(R555E) blocked this effect of NGF. Taken together, these data indicate that SH2-B is a positive regulator of NGF-mediated activation of the Akt/Forkhead pathway.

Introduction

Nerve growth factor (NGF) is a trophic factor essential for the development and survival of sympathetic and sensory neurons. PC12 cells, a well-established cell culture model of sympathetic neurons, differentiate into a neuronal-like phenotype in the presence of NGF[1]. In both sympathetic neurons and PC12 cells, removal of trophic support triggers retraction of neurite outgrowths and eventual apoptosis (107, 108). NGF protects PC12 cells from apoptosis induced by trophic factor withdrawal (108, 109), cytotoxic drugs (110-112), and oxidative stressors (113-115). The mechanism(s) by which NGF elicits its effects on neuronal differentiation and survival are only beginning to be understood.

A majority of the neurotrophic effects of NGF are believed to be initiated by binding of NGF to the membrane receptor tyrosine kinase TrkA. NGF binding activates TrkA which phosphorylates itself on multiple tyrosines (126). Binding of different signaling proteins to these phosphorylated tyrosines initiates multiple signaling pathways. Several of these TrkA binding proteins and/or their downstream effector proteins have been implicated in the regulation of neuronal differentiation and/or survival. One of the first pathways shown to be required for NGF-induced neuronal differentiation of PC12 cells is the Ras/Raf/MEK/ERK pathway. The TrkA binding protein Shc, as well as Ras, Raf, MEK and ERK have been implicated in neuronal differentiation of PC12 cells (28, 29, 127, 128). Another TrkA binding protein implicated in NGF-induced differentiation of PC12 cells is phospholipase Cγ, a protein that mediates the production of diacylglycerol and inositol trisphosphate, leading to release of intracellular Ca^{2+} stores and activation of protein kinase C (129-131).

A third pathway, involving the TrkA binding protein phosphatidylinositol 3' (PI3) kinase and the serine/threonine kinase Akt/PKB, has been implicated in axon caliber and branching (132). However, it is primarily emerging as a transducer of

survival signals. Akt activity has been linked to cell survival in several cell types (133, 134) and is both necessary and sufficient for NGF-dependent survival of sympathetic neurons (135). Inhibition of PI3 kinase by wortmannnin or LY294002 blocks the ability of NGF to prevent apoptosis in both PC12 cells and primary sympathetic neurons (135, 136). Activated forms of PI3 kinase and AKT promote neuronal survival in the absence of external survival factors (134, 135, 137-142). Lipid products of PI3 kinase have been shown to recruit Akt to the plasma membrane via the PH domain of Akt, leading to phosphorylation of Akt at Ser473 and Thr308 by phosphoinositide-dependent kinases (143-145). Phosphorylation of both sites is required for full activation of Akt (146). Akt can phosphorylate and modulate the activity of several proteins involved in cellular survival, including members of the FOXO family of forkhead transcription factors (147, 148), glycogen synthase kinase-3 (GSK-3) (149), the Bcl2 family members BAD and Bax (150-152), nuclear factor-κB (153, 154) and caspase 9 (155). Phosphorylation of GSK-3 by Akt at Ser 21 (α isoform) or Ser 9 (β isoform) inactivates GSK-3 and is believed to be the primary mechanism responsible for growth factor inhibition of this protein kinase(156, 157). Inactivation of GSK-3 is thought to contribute to the survival effects of Akt activation and to reduce neurotrophin factor withdrawal-induced neurite retraction. Inhibition of GSK-3 by LiCl reduces NGF withdrawal-induced apoptosis in PC12 cells (158) and the degree of neurite retraction observed in wortmannnin-treated SH-SY5Y neuroblastoma cells (159).

Multiple Forkhead family members are targets of Akt (148, 160). Three mammalian members of this family, namely FKHR/FOXO1 (161), FKHRL1/FOXO3 (162) and AFX/FOXO4 (163), belong to the FOXO subfamily. Proteins in this subfamily have sequence similarity to the nematode homologue Daf-16, which is a downstream target of two Akt homologues in an insulin-related signaling pathway, and have been implicated in apoptosis of neuronal cells (164-166). Daf-16 as well as AFX, FKHR, and FKHRL1 contain multiple consensus

Akt phosphorylation sites (161, 163) and Akt can directly phosphorylate the three mammalian forkhead proteins in vitro (148, 160).

Akt is thought to inhibit the activity of forkhead transcription factors primarily by regulating their subcellular localization (117, 167). In this paradigm, FKHR in the nucleus induces the expression of genes critical for cell death, such as the Fas ligand gene. When activated, Akt phosphorylates FKHR proteins in the nucleus, resulting in binding of FKHR proteins to 14-3-3 and subsequent export from the nucleus. This relocation of FKHR proteins to the cytoplasm functionally represses their transcriptional activity (117, 118, 167, 168). Binding to 14-3-3 is also thought to help retain FKHR in the cytoplasm by masking a site necessary for nuclear reimport (118).

SH2-B was identified as a binding protein of the receptor for NGF (TrkA) (33, 34), as well as of the receptors for insulin (36), platelet-derived growth factor (39), fibroblast growth factor (58), insulin like growth factor-1 (38), hepatocyte growth factor (58); the cytokine receptor-associated tyrosine kinase JAK2 (41), and the R1 Fc epsilon R1 receptor (43). Four SH2-B isoforms (α, β, γ, δ) have now been identified; they differ in their C-termini downstream of the SH2 domain (37, 41, 43, 46) (FIG. 1.1). SH2-B belongs to a family of adapter proteins that include APS and Lnk (44, 45).

We and others have shown that SH2-B (α or β isoform) is vital for NGF-induced neurite outgrowth in cultured PC12 cells and maintenance of the neuronal phenotype of primary cultured rat sympathetic neurons (33, 34). NGF stimulates the association of SH2-Bβ with TrkA via the SH2 domain of SH2-Bβ and the tyrosyl phosphorylation of SH2-Bβ. Mutating the critical Arg to Glu (R555E) within the SH2 domain of SH2-Bβ abolishes both the NGF-induced association of SH2-Bβ with TrkA and the tyrosyl phosphorylation of SH2-Bβ (33). Stable overexpression of SH2-Bβ enhances NGF-induced neuronal differentiation of PC12 cells, whereas stable expression of SH2-Bβ(R555E) blocks NGF-induced differentiation of PC12 cells (33). Interestingly, cells stably expressing

SH2-Bβ(R555E) do not show impaired NGF-induced tyrosyl phosphorylation of TrkA, Shc, phospholipase C-γ, ERK1 or ERK2 (41), suggesting that impaired activation of these signaling proteins is not responsible for the dramatic overall impairment of neuronal differentiation. SH2-B has also been implicated in neuronal survival. Neuronal sympathetic neurons triturated with anti-SH2-B antibodies exhibit a reduced rate of survival when grown in NGF-containing medium. Similarly, transient expression of an N-terminally truncated form of SH2-B promotes degeneration of axons of sympathetic neurons in explants of superior cervical ganglia grown in the presence of NGF (34). In the current work, we extend these earlier studies designed to determine the role of SH2-Bβ in neuronal differentiation and survival by examining the hypothesis that SH2-Bβ positively regulates NGF-induced activation of the protein kinase Akt and its downstream targets.

Materials and Methods

Cells and Reagents. Parental PC12 cells were obtained from Dr. Ben Margolis (University of Michigan) or ATCC. Pools of PC12 cells stably expressing GFP, GFP-SH2-Bβ and GFP-SH2-Bβ(R555E) were made as described previously (41) and used for FIGS. 2.2-2.5. FIG. 2.8 used cells made in a similar fashion with the exception that all GFP positive cells were pooled instead of only those with the top 2% expression level. The stock of 293T cells was obtained from Dr. O. A MacDougald (University of Michigan, Ann Arbor, MI). Murine NGF was from BD Bioscience. Triton X-100, aprotinin, and leupeptin were purchased from Roche Molecular Biochemicals. The nitrocellulose membranes and enhanced chemiluminescence (ECL) detection system were from Amersham Corp. X-ray film came from DuPont or Kodak (FIG. 2.8B), horse serum from ICN Biomedicals, fetal bovine serum from Invitrogen and poly-L-lysine from Sigma.

Plasmids, Antibodies and Fluorescent Probes. pcDNA-Flag-FKHR was a gift of Drs. E. Tang and K. Guan (148). cDNAs encoding GFP-tagged wild-type SH2-Bβ and SH2-Bβ(R555E) have been described previously (33). Anti-SH2-Bβ antibody (αSH2-Bβ) was prepared as described previously (41) and used at a dilution of 1:15,000 for western blotting. Antibodies that recognize the following proteins were used for western blotting at a dilution of 1:1000: phospho-Akt (Ser473) (αpAkt-Ser473) (Cell Signaling, #9276), Akt (αAkt) (Cell Signaling, #9272), FKHRL1 (αFKHRL1) (Upstate Biotechnology, #06-951), phospho-GSK-3(Ser 21/9) (αpGSK-3) (Cell Signaling, #9331S) and phospho-GSK-3(Y279/Y216) (used to detect total GSK-3) (αGSK-3) (Upstate Biotechnology, #05-413). Anti-Flag M2 monoclonal antibody (αFlag) was obtained from Sigma and used at a dilution of 1:1000 for immunocytochemistry. Texas Red dye-conjugated AffiniPure Goat Anti-mouse IgG (H+L) was from Jackson

ImmunoResearch and was used at a dilution of 1:400. Anti-rabbit IgG conjugated to horseradish peroxidase was from Santa Cruz. Antibody to FKHR (αFKHR)(Cell Signaling, 9462) which recognizes only unphosphorylated FKHR (T Maures and C Carter-Su, unpublished observation and personal communication with Cell Signaling technical support)

Cell Culture. PC12 cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM L-glutamine, and 1 mM antibiotic-antimycotic (Invitrogen) (supplemented DMEM), 10% heat-inactivated horse serum, and 5% fetal bovine serum. Cells used for experiments were plated on poly-L-lysine coated coverslips (for analysis by confocal microscopy), Lab-TekII Chamber Slide System 154941 (Nalge Nunc International Corp) (FIG. 2.8A) or on collagen-coated dishes. The latter were prepared by incubating with 0.1mg/ml collagen (type I rat tail, Collaborative Biomedical Products, #354236) in 0.02N acetic acid for 1 h. The confluent cells were deprived of serum overnight using supplemented DMEM containing 1% bovine serum albumin and treated as indicated with NGF. 293T and Cos-7 cells were grown in supplemented DMEM and 8% calf serum.

RT-PCR. Total RNA was prepared from PC12 cells using TRIZOL reagent as per the manufacturer's protocol (Life Technologies). Oligonucleotide primers that recognize all four known isoforms of murine SH2-B were used to amplify 1 μg of total RNA (sense primer 5'- TCTCCCCTAGTTCTGCCTCCATTG-3', corresponding to nucleotides 1638-1661 of murine SH2-Bα, β, γ, δ; antisense primer 5'-CGCCCCCGACGCCTCTTCT-3' corresponding to nucleotides 2428-2410 in murine SH2-Bγ, Genebank accession# AF421139 (46). Expression of SH2-Bγ was determined using the sense primer 5'-TTCAACTTCCAGGGCAAGGCTAA-3' (corresponding to the rat equivalent of nucleotides 1991-2013 in murine SH2-Bα, β, γ, δ in which nucleotide 2011 is a T rather than an A) and an antisense primer specific for SH2-Bγ (5'-CCGGCCTCACTTCTTGGGTGCA-3', corresponding to nucleotides 2220-2241 in

murine SH2-Bγ). Reaction products were resolved by agarose gel electrophoresis and detected by ethidium bromide staining. PCR products were extracted (Qiagen Qiaex II Gel Extraction Kit) and sequenced to verify which isoform they represented.

Immunoblotting. Cells were solubilized in lysis buffer (50mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2mM EGTA). The lysates were centrifuged and the supernatants boiled for 5 min in a mixture (80:20) of lysis buffer:sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Solubilized proteins were separated by SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with desired antibodies and enhanced chemiluminescence (41). For quantification, immunoblots with signals in the linear range were scanned and the resulting images were analyzed using multi-analyst image software from Bio-Rad (FIG. 2.3, 2.4C). In FIG. 2.5B quantification used the NIH Image 1.62 f software.

Immunolocalization. PC12 cells were plated onto poly-L-lysine treated glass coverslips or Lab-TekII Chamber Slide System and transfected with various cDNA using Lipofectamine Plus (Invitrogen). Both 293T and Cos-7 cells were plated on coverslips and transfected using calcium phosphate precipitation. Twenty-four h after transfection, cells were incubated in serum-free medium overnight. As indicated, NGF was added following serum deprivation at a concentration of 100ng/ml. Cells were then fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with αFlag for 1 h, then for 45 min with anti-mouse IgG conjugated to Texas Red. Coverslips were mounted using mounting medium (90% glycerol and 10% phosphate buffered saline) (FIGS. 2.6B, 2.7B, 2.7C) or Prolong Anti-fade P-7481 (Molecular Probes) (FIGS. 2.7A, 2.8A) and examined using epifluorescence microscopy (Nikon Eclipse TE 200) with a 60x objective. In other experiments, coverslips under a Noran OZ laser scanning confocal microscope equipped with a 100x Nikon objective. In some experiments, fluorescently labeled antibodies and GFP-

tagged proteins were imaged Where indicated, the mean intensities of neighboring cytosolic and nuclear regions (approximately 5 μm^2 each) were calculated using Adobe Photoshop™, corrected for background, and presented as nuclear-to-cytosol fluorescence ratios.

In Vitro Akt Kinase Assays. Immunocomplex Akt kinase assays were performed using the Akt1/PKB alpha IP-kinase assay kit from Upstate Biotechnology. After 4-5 h serum deprivation, NGF was added to PC12 cells stably expressing GFP, GFP-SH2-Bβ or GFP-SH2-Bβ(R555E) for various lengths of time. PC12 cells were placed on ice and proteins were extracted according to the manufacturer's instructions. Akt was immunoprecipitated from cell lysates using anti-Akt/PKB antibody. After washing, the immunoprecipitates were resuspended in 40 μl of kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP and 10μl of a specific substrate peptide (RPRAATF) and incubated at room temperature for 20 min with constant shaking. The reaction was stopped by addition of 20 μl of 40% trichloroacetic acid. The phosphorylated substrate was spotted onto P81 phosphocellulose paper (Whatman). Residual [γ-^{32}P]ATP was removed by extensive washing. Radioactivity bound to the filter was quantified using a scintillation counter.

Results

Isoforms of SH2-B Expressed in PC12 Cells. We first examined which isoforms of SH2-B are predominantly expressed in PC12 cells. RNA prepared from PC12 cells was PCR amplified using primers that would amplify all four known isoforms (α, β, γ, δ) (predicted products: ~637, 737, 790, 690 bp respectively) (FIG. 2.1A). The PCR products migrated with a size most closely resembling that predicted for the β isoform (FIG. 2.1B, lane 1) and co-migrated with the PCR product using SH2-Bβ cDNA as template (data not shown). The PCR products were cleaved by the restriction enzyme BsiEI which cleaves only the 100 bp insert present in the β and γ isoforms (FIG. 2.1A). The BsiEI products (FIG. 2.1B, lane 3) co-migrated with the BsiEI cleaved forms of the PCR product obtained using rat SH2-Bβ cDNA as the template (FIG. 2.1B, lane 4). To confirm the identity of the PCR product as SH2-Bβ, the PCR product was gel purified and sequenced. The sequence corresponded to that predicted for the β isoform, suggesting that SH2-Bβ is the primary isoform of SH2-B expressed in PC12 cells. Because the RT-PCR products for the β and γ isoforms are only 53 bp different in size and therefore might be difficult to separate on the gel and because the expression levels of these two isoforms could be very different, PCR primers

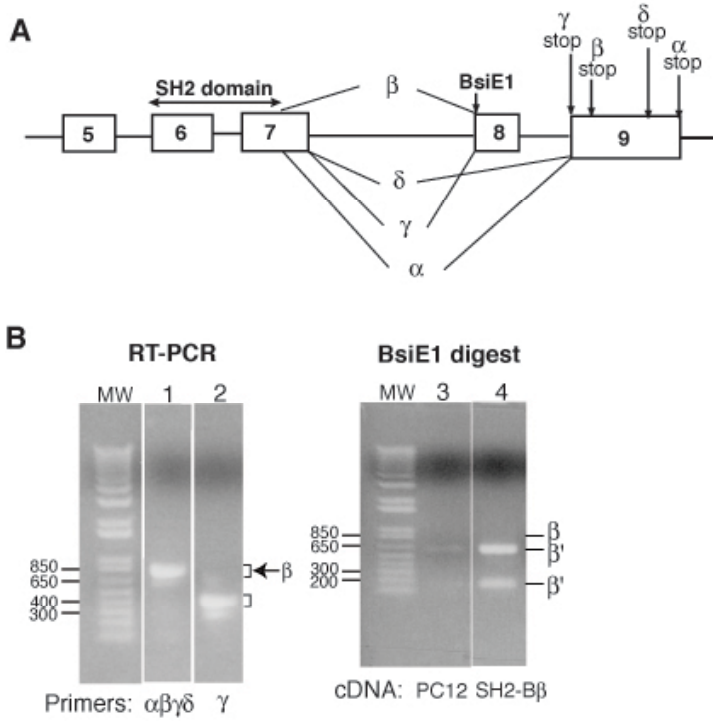


FIG. 2.1. PCR reveals the expression of the β isoform of SH2-B in PC12 cells.

A) Schematic of the 4 isoforms of SH2-B. Exons are denoted by the boxes and are numbered. B) Total RNA was prepared from cells. Oligonucleotide primers that recognize all four known isoforms of murine SH2-B (lane 1) or the γ isoform (lane 2) were used to amplify 1 μg of total RNA using PCR. The PCR product from a duplicate of lane 2 (lane 3) or the PCR product from cDNA encoding SH2-Bβ (lane 4) was digested with BsiE1 and digestion products separated in an agarose gel. For lanes 3 and 4, β denotes the predicted migration of the PCR product using the β isoform as template and β' denotes the predicted migration of the BsiE1 digestion products of the β isoform.

specific for the γ isoform of SH2-B were designed to test further whether SH2-Bγ is expressed in PC12 cells. Figure 1B (lane 2) shows that the γ isoform is also expressed in PC12 cells. Since it was possible that some δ isoform was also amplified by the primers used even though the primer sequence was not an exact match, the PCR amplified cDNA using γ specific primers was gel purified and sequenced. The sequence obtained from two different PCR products corresponded to the γ isoform. Taken together, the results of FIG. 2.1 are consistent with SH2-Bβ being the primary isoform of SH2-B expressed in PC12 cells. Other isoforms (e.g. γ isoform) appear to be expressed, but to a significantly lesser extent than the β isoform. These results are consistent with the previous finding that after dephosphorylation by alkaline phosphatase, SH-2B in PC12 cells comigrates with the dephosphorylated SH2-Bβ expressed ectopically in COS cells (33). Based upon these findings, all subsequent experiments focused on the β isoform of SH2-B.

SH2-Bβ Enhances NGF Stimulated Activation of Akt. To determine whether SH2-Bβ is involved in the regulation of Akt by NGF, we first examined whether stable overexpression of SH2-Bβ or a mutated form of SH2-Bβ that lacks a functional SH2 domain, SH2-Bβ(R555E), affects the ability of NGF to stimulate the phosphorylation of Serine 473 in Akt. Maximal activation of Akt requires phosphorylation of Ser473 (146). PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) were treated with 100 ng/ml NGF for various times and cell lysates were collected. Solubilized proteins were separated by SDS-PAGE and immunoblotted with antibody that specifically recognizes phosphorylated Ser473 (pSer473) within Akt. In control cells overexpressing GFP, NGF induced the appearance of pSer473 within Akt within 15 min (FIG. 2.2A, top panel, lane 2). Phosphorylation was maximal at the 15 min timepoint and returned to nondetectable levels by 2 h. A similar NGF-induced increase in the amount of pSer 473, both in terms of magnitude and time course, was observed in cells overexpressing GFP-SH2-Bβ (R555E) (FIG. 2.2A, bottom

panel). In contrast, overexpression of GFP-SH2-Bβ both enhanced and prolonged NGF-induced phosphorylation of Ser473 (FIG. 2A, middle panel). The observed changes in the amount of pSer473 were not due to changes in the amount of Akt. No significant change in total Akt was observed after NGF treatment and the levels of Akt in the three cell lines were indistinguishable (FIG. 2.2B). These observations demonstrate that overexpression of SH2-Bβ enhances NGF induced Akt activation while overexpression of SH2-Bβ (R555E) has no effect. To verify levels of expression of GFP-SH2-Bβ in the stably expressing cell lines, cell lysates were immunoblotted with antibody to SH2-B (αSH2-B). FIG. 2.2C shows a very high level of expression (>10 fold) of both GFP-SH2-Bβ and GFP- SH2-Bβ(R555E) compared to endogenous SH2-Bβ. These levels of expression of SH2-Bβ are sufficiently high to enhance (GFP-SH2-Bβ) or block (GFP-SH2-Bβ(R555E), NGF-induced neuronal differentiation of PC12 cells ((33); data not shown).

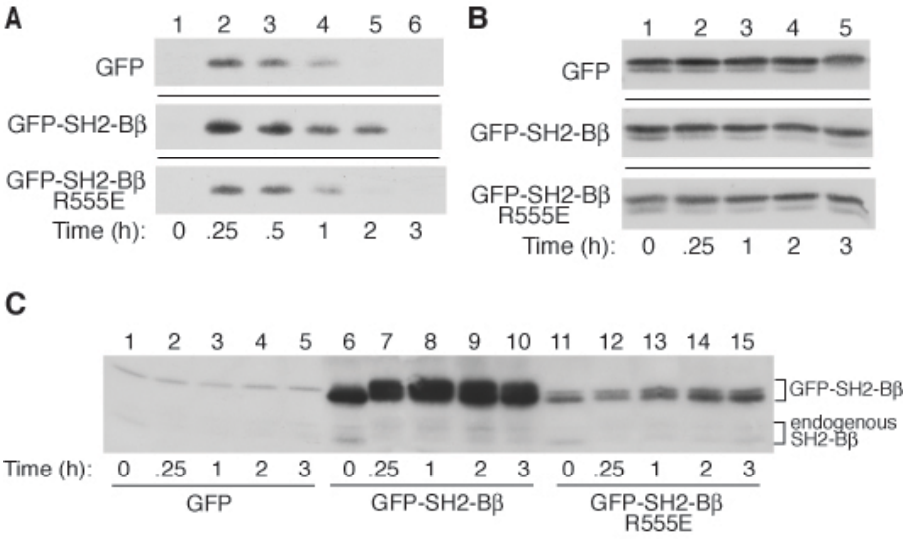


FIG. 2.2. SH2-Bβ enhances and prolongs NGF-induced phosphorylation of Ser473 in Akt.

PC12 cells stably overexpressing GFP alone (GFP), GFP-SH2-Bβ or GFP-SH2-Bβ (R555E) as indicated were stimulated with 100 ng/ml NGF for the indicated times. In parallel experiments, an equal amount of protein in the lysates was immunoblotted with αpAkt(Ser473) (Panel A), αAkt (Panel B), or αSH2-B (Panel C). SH2-Bβ(R555E) compared to endogenous SH2-Bβ. These levels of expression of SH2-Bβ are sufficiently high to enhance (GFP-SH2-Bβ) or block (GFP-SH2-Bβ(R555E), NGF-induced neuronal differentiation of PC12 cells ((33); data not shown).

To verify more directly the effect of SH2-Bβ on NGF-induced activation of Akt, we measured the enzymatic activity of Akt following exposure of the different cell lines to NGF. Cells were treated with NGF and solubilized. Akt was immunoprecipitated using anti-Akt/PKB antibody (αAkt) and incubated at room temperature for 20 min with [γ-^{32}P]ATP and a specific substrate peptide (RPRAATF). The reaction was stopped by addition of trichloroacetic acid and the phosphorylated substrate was spotted onto P81 phosphocellulose paper. Radioactivity bound to the filter was quantified by scintillation counting. The time course of Akt activation in response to 100 ng/ml NGF generally paralleled that of phosphorylation of Akt on Ser473. In control PC12 cells stably expressing GFP alone, stimulation with NGF resulted in a dramatic increase in Akt activity after 3 min. Akt activity returned to near baseline levels by 2 h (FIG. 2.3A). Similar results were obtained in PC12 cells stably expressing SH2-Bβ(R555E) (data not shown). In contrast, but consistent with the pSer473 results, Akt activity in PC12 cells stably expressing GFP-SH2-Bβ was enhanced at 15 and 60 min compared to control cells expressing GFP and remained elevated for 2 h. Overexpression of GFP-SH2-Bβ did not enhance the activity of Akt at the three min time point. However, overexpression of GFP-SH2-Bβ was found in preliminary studies to enhance NGF-induced Akt activity at the 3 min time-point when NGF was tested at a concentration of 10 or 50 ng/ml, rather than at 100 ng/ml (data not shown), suggesting that SH2-Bβ not only prolonged NGF-activation of Akt but also shifted the peak response to an earlier time point.

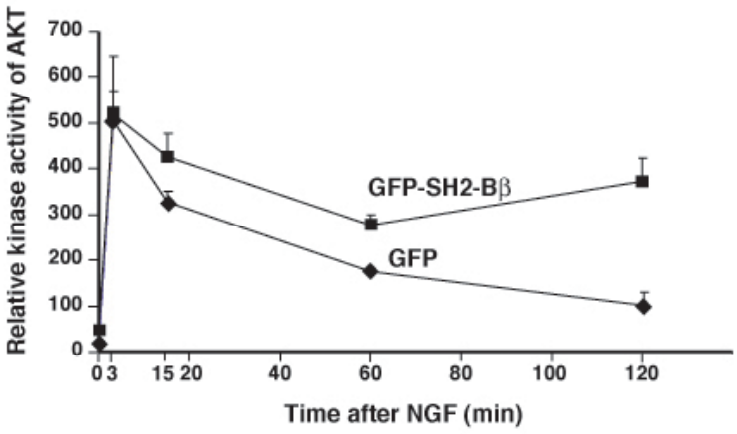


FIG. 2.3. SH2-Bβ enhances and prolongs NGF-induced activity of Akt.
PC12 cells stably overexpressing GFP alone and GFP-SH2-Bβ were stimulated with 100 ng/ml NGF for the indicated times. Akt was immunoprecipitated and kinase activity was determined. The results show the mean and range of two separate experiments.

SH2-Bβ Enhances Phosphorylation of GSK-3. To investigate whether SH2-Bβ affects downstream targets of Akt, we first examined whether overexpression of SH2-Bβ alters NGF-induced phosphorylation of GSK-3 on Ser-21 (α isoform) or Ser-9 (β isoform). Akt phosphorylation of the GSK-3α isoform on Ser-21 or of GSK-3β on Ser-9 results in partial inactivation of GSK-3 (157). Phosphorylation of both GSK-3α and GSK-3β in response to NGF was rapid, substantial and transient, with maximal stimulation being observed at 15 min or 1 h (FIGS. 2.4A and C). As with Akt, phosphorylation of GSK-3 was prolonged in PC12 cells stably expressing SH2-Bβ compared to cells expressing GFP alone or SH2-Bβ(R555E). The observed changes in the amount of pSer21/9 were not due to changes in the amount of GSK-3. No significant change in total GSK-3α/β was observed after NGF treatment and the levels of GSK-3 in the three cell lines were indistinguishable (FIG. 2.4B).

SH2-Bβ Enhances NGF-induced Phosphorylation of FKHRL1. To investigate further whether SH2-Bβ affects downstream targets of Akt, we examined whether SH2-Bβ affects NGF induced phosphorylation of forkhead (FKHR/FOXO) family members. As shown in the anti-FKHRL1 (αFKHRL1) immunoblots in FIG. 2.5A (upper panel), in control cells overexpressing GFP alone, NGF induces a transient upward shift in the migration of FKHRL1, consistent with an increase in phosphorylation. The ratio of phosphorylated (upper band) to unphosphorylated (lower band) FKHRL1 was maximal at 15 or 30 min and declined thereafter (Fig 5A, 5B). Phosphorylation was significantly prolonged in PC12 cells stably expressing SH2-Bβ (FIG. 2.5A, middle panel) compared to control cells expressing GFP alone (FIG. 2.5A, upper panel) or cells expressing SH2-Bβ(R555E) (FIG. 2.5A, lower panel).

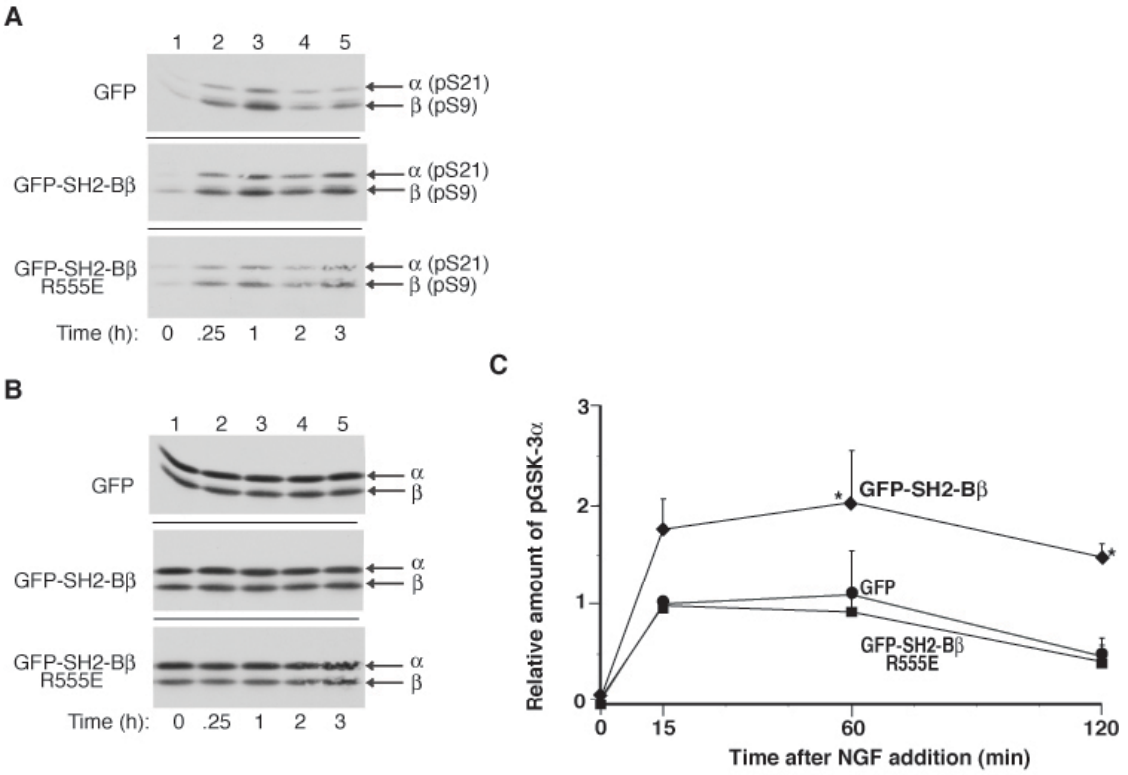


FIG. 2.4. SH2-Bβ enhances and prolongs NGF-induced phosphorylation of GSK-3α/β on Ser21/Ser9.

PC12 cells stably overexpressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ (R555E) were stimulated with 100 ng/ml NGF for the indicated times. An equal amount of protein in the lysates was immunoblotted with αpGSK-3(Ser 21/9) (Panel A) or αGSK-3 (Panel B). The resulting films from three separate experiments using αpGSK-3 were scanned and the relative amounts of bound pGSK-3 were calculated (Panel C). The results show the mean and standard error of the mean for three separate experiments, normalized to the 15 min control (GFP). An asterisk (*) denotes a p value <0.05 (two-tailed, paired Student t-Test) between experimental [GFP-SH2-Bβ and GFP-SH2-Bβ (R555E)] and control (GFP) values.

SH2-Bβ(R555E) Redistributes FKHR to the Nucleus. FKHR transcription factors are inactivated by Akt-mediated phosphorylation which stimulates the redistribution of FKHR from the nucleus to the cytoplasm (117, 167, 168). Because SH2-Bβ stimulated the NGF-induced phosphorylation of FKHRL1, we examined whether overexpression of SH2-Bβ would alter the subcellular distribution of FKHR proteins. PC12 cells were transiently co-transfected with cDNAs encoding Flag-tagged FKHR and either GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E). After 24 h, the cells were incubated in serum-free medium, fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with αFlag followed by anti-mouse IgG conjugated to Texas Red. In some experiments, the location of Flag-FKHR was determined in cells that co-expressed GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E) using a Noran OZ laser scanning confocal microscope equipped with a 100× Nikon objective (FIG. 2.6A, upper panel). In other experiments, GFP and fluorophore conjugated antibodies were imaged by epifluorescence microscopy (Nikon Eclipse TE 200) with a 60x objective (FIG. 2.6A, lower panel). The mean intensities of neighboring cytosolic and nuclear regions (approximately 5 μm2 each) in the epifluorescent images were calculated using Adobe PhotoshopTM, corrected for background, and expressed as nuclear-to-cytosol fluorescence ratios (FIG. 2.6B). FIGS 2.6A and B show that in control PC12 cells co-expressing GFP and FKHR under quiescent conditions, FKHR was mainly localized in the cytoplasm and excluded from the nucleus. Co-expression of GFP-SH2-Bβ did not generally alter the subcellular localization of FKHR in these cells. In contrast, overexpression of SH2-Bβ (R555E) caused a dramatic redistribution of FKHR to the nucleus.

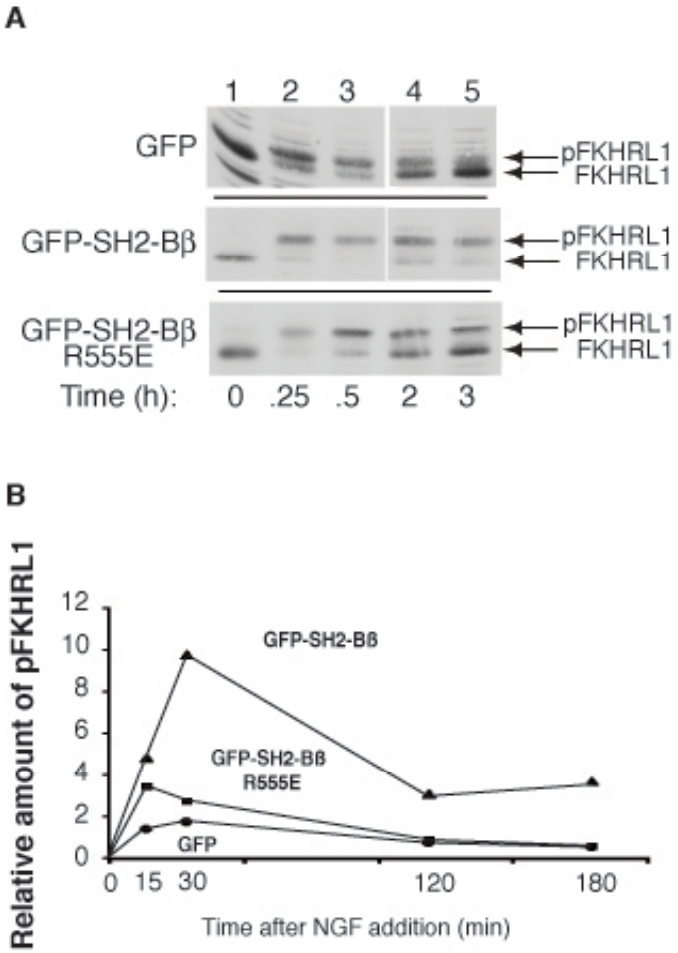


FIG. 2.5. SH2-Bβ enhances and prolongs NGF-induced phosphorylation of FKHRL1.

PC12 cells stably overexpressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ (R555E) were stimulated with 100 ng/ml NGF for the indicated times. An equal amount of protein in the lysates was immunoblotted with αFKHRL1 (Panel A). The films in Panel A were scanned and the relative amounts of FKHRL1 were calculated (Panel B). The results show the mean intensity of the phosphorylated FKHRL1 band (upper band) divided by the unphosphorylated FKHRL1 band (lower band).

To determine whether this ability of SH2-Bβ (R555E) to redistribute FKHR was specific to PC12 cells, we examined the effect of expressing GFP, GFP-SH2-Bβ, and GFP-SH2-Bβ (R555E) on the subcellular distribution of Flag-FKHR in cultured Cos-7 and 293T cells. In transiently transfected Cos-7 cells (FIG. 2.7A), Flag-FKHR was predominantly localized in the cytoplasm and excluded from the nucleus of about 75% of Cos-7 cells expressing GFP. Much like what was observed in the PC12 cells, co-expression of GFP-SH2-Bβ with Flag-FKHR did not alter the subcellular localization of FKHR. However, overexpression of SH2- Bβ (R555E) resulted in increased retention (from ~25% to ~40% of cells) of FKHR within the nucleus of Cos-7 cells (40%). 293T cells ectopically expressing Flag-FKHR and GFP displayed a lower basal level of cytoplasmic FKHR (~50%) and a higher basal level of nuclear FKHR than seen in Cos-7 cells (Fig 7B, C). In these cells, overexpression of SH2-Bβ caused an increase in the percentage of the cells (~65%) that retained FKHR in the cytoplasm and excluded it from the nucleus. As observed with PC12 and Cos-7 cells, ectopic expression of SH2-Bβ (R555E) caused a substantial decrease in the percentage of 293T cells containing FKHR in the cytoplasm (from ~50% to ~30%) and corresponding increase in the percentage of cells containing FKHR in the nucleus (from ~50% to ~70%).

Thus, a substantial percentage of all three cell types exhibited a subcellular redistribution of FKHR from the cytoplasm to the nucleus when expressing SH2-Bβ (R555E). To determine whether this nuclear localization could be reversed with NGF stimulation, PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E) were transiently transfected with cDNA encoding Flag-FKHR. After transfection the cells were deprived of serum overnight and treated with 100 ng/ml NGF for 1 h before fixing and staining. Figure 8 demonstrates FKHR's characteristic subcellular shift after NGF treatment in the GFP expressing control cells. The addition of NGF led to an

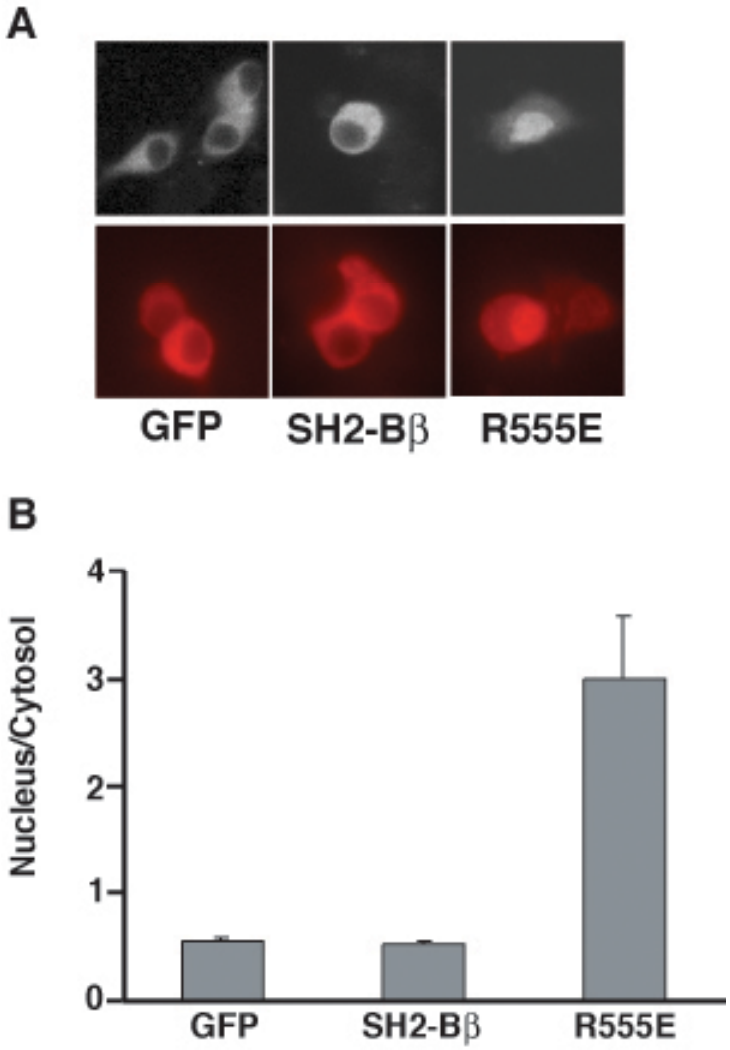


FIG. 2.6. SH2-Bβ (R555E) promotes FKHR retention in the nucleus of PC12 cells.

PC12 cells (Panels A and B) were transiently cotransfected with cDNA encoding Flag-FKHR and either GFP alone, GFP-SH2-Bβ or GFP-SH2-Bβ (R555E). Ectopically expressed proteins were imaged using either confocal (Panel A, upper) or epifluorescence (Panel A, lower) microscopy. For Panel B, relative levels of FKHR in the cytoplasm and nucleus were determined by comparing the intensity of the signal in identically sized regions placed in the cytoplasm and nucleus of the same cell (Panel B). The results show the mean and standard error for a total of 16-18 cells from experiments performed on 3-5 separate days.

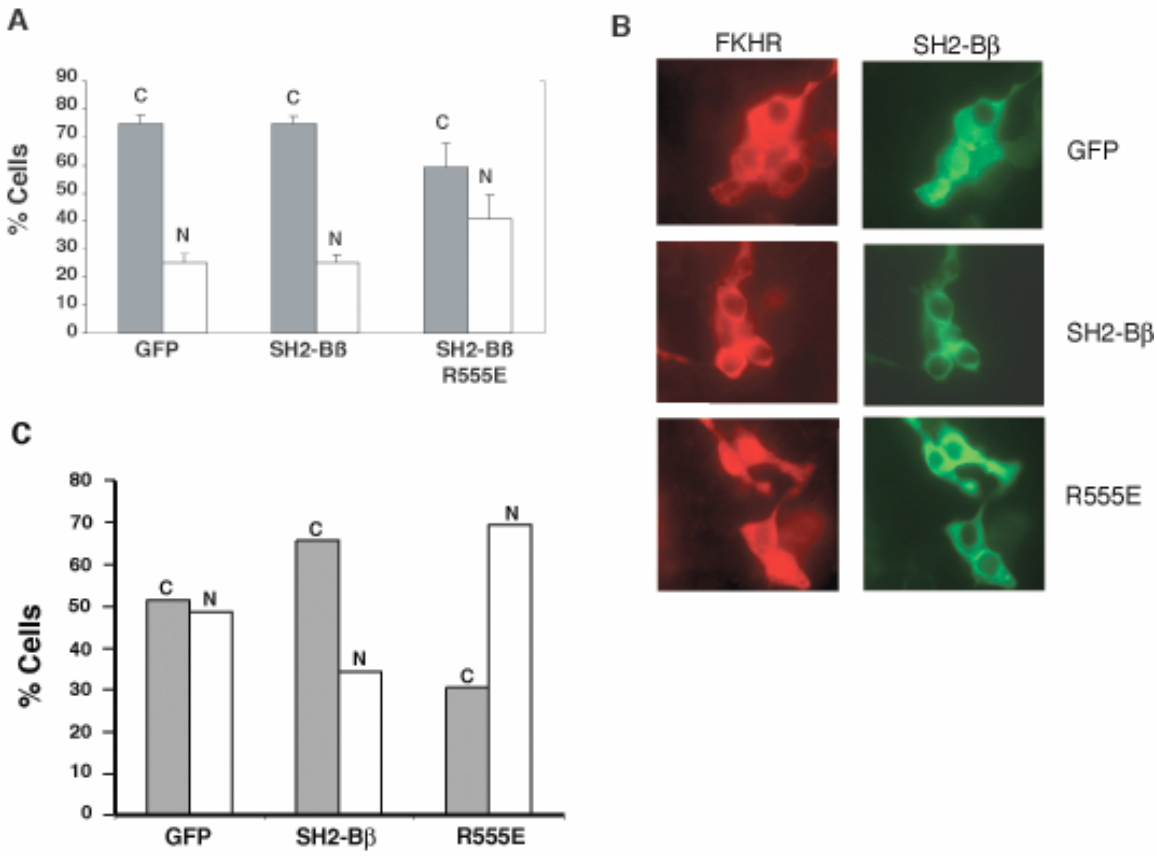


FIG. 2.7. SH2-Bβ(R555E) redistributes FKHR to the nucleus in 293T and Cos-7 cells.

A) Cos-7 cells were transiently cotransfected with cDNA encoding Flag-FKHR and either GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E). The number of COS-7 cells in which FKHR was excluded from the nucleus (designated "C for "cytoplasmic") and in which FKHR was present or concentrated in the nucleus (designated "N") were graphed as a percentage of the total number of GFP-positive cells counted. 116, 69, and 103 cells expressing GFP alone, GFP-SH2-Bβ, and GFP-SH2-Bβ (R555E) respectively were assessed from two trials. B and C) 293T cells were transiently cotransfected with cDNA encoding Flag-FKHR and either GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E). Ectopically expressed proteins were imaged using epifluorescence microscopy (Panel B). The number of 293T cells in which FKHR was excluded from the nucleus (designated "C for "cytoplasmic") and in which FKHR was present or concentrated in the nucleus (designated "N") were graphed as a percentage of the total number of GFP-positive cells counted. 33, 35 and 49 cells expressing GFP alone, GFP-SH2-Bβ or GFP-SH2-Bβ(R555E) respectively were assessed.

increased number of cells with Flag-FKHR excluded from the nucleus (from ~40% to ~60%). Consistent with the studies using 293T cells which displayed a similar percentage of cells in which FKHR was excluded from the nucleus, stable expression of GFP-SH2-Bβ in PC12 cells increased the number of cells with cytoplasmic Flag-FKHR, to a level above that seen in the control cells even in the presence of NGF. Treatment with NGF did not result in a further increase in the number of cells with FKHR in the cytoplasm. In contrast, PC12 cells stably expressing GFP-SH2-Bβ (R555E) were unable to shift FKHR from the nucleus to the cytoplasm even after NGF treatment. These results suggest that overexpression of SH2-Bβ can mimic the action of NGF on the subcellular distribution of FKHR whereas overexpression of SH2-Bβ(R555E) blocks the ability of NGF to redistribute FKHR to the cytoplasm.

Because cytoplasmic localization of FKHR is thought to be dependent on its phosphorylation, we examined whether overexpression of SH2-Bβ or SH2-Bβ (R555E) affects the serine/threonine phosphorylation of FKHR measured under the exact experimental conditions used to study the subcellular localization of FKHR in FIG. 2.8A. Cells were deprived and stimulated with NGF in the same manner as above, before collecting total cell lysates. Proteins were separated by SDS-PAGE and immunoblotted with anti-FKHR, an antibody that recognizes unphosphorylated but not phosphorylated FKHR. Thus, the degree of phosphorylation is indicated by a decrease in the signal. As shown in Fig 8B, phosphorylation of ectopically expressed Flag-FKHR was identical in cells expressing GFP and SH2-Bβ (R555E). Phosphorylated Flag-FKHR peaked between 15-30 minutes in both lines and began to return to its unphosphorylated form by 2 h. Also, consistent with the FKHRL1 results (Fig 5 A, B), cells

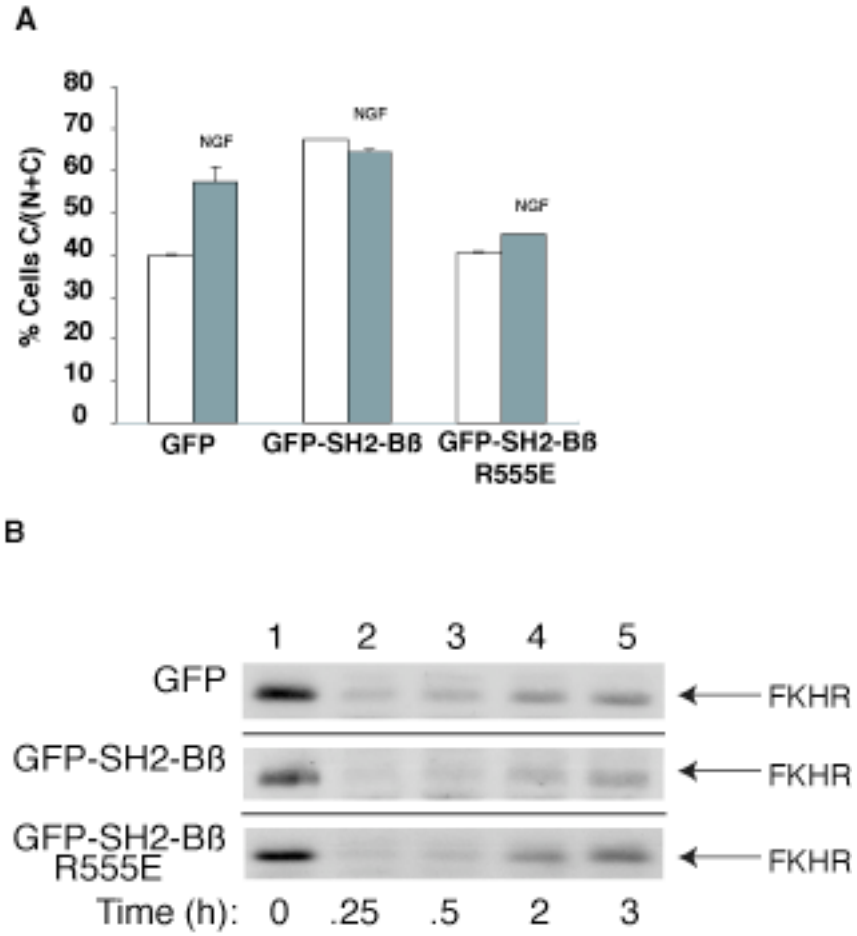


FIG. 2.8. SH2-Bβ mimics and SH2-Bβ(R555E) blocks NGF-induced redistribution of FKHR from the nucleus to the cytoplasm.

PC12 cells stably expressing GFP, GFP-SH2-Bβ, or GFP-SH2-Bβ (R555E) were transiently transfected with cDNA encoding Flag-FKHR. A) Cells were incubated in serum free medium for 24 h and fixed (white bars) or treated with 100ng/ml NGF for 1 h and fixed (gray bars). Units are defined as the percentage of cells with Flag-FKHR excluded from the nucleus (defined "C" for "cytoplasmic") divided by the total number of cells counted of which expressed Flag-FKHR (defined "N+C"). 103, 100, 114, 192, 126, and 107 cells expressing GFP alone (minus and plus NGF), GFP-SH2-Bβ (minus and plus NGF), and GFP-SH2-Bβ (R555E) (minus and plus NGF) respectively were assessed in two trials. B) Cells were deprived for 24 h and then treated with 100ng/ml NGF for 0, 0.25, 0.5, 2, and 3 h. An equal amount of protein in the lysates was immunoblotted with αFKHR. It is important to note that this antibody recognizes only unphosphorylated FKHR.

expressing GFP-SH2-Bβ showed an increase in the duration of FKHR phosphorylation. Similar results were obtained looking at the phosphorylation of endogenous FKHR both in cells that did and cells that did not co-express Flag-FKHR (data not shown). Thus, FKHR appears to be phosphorylated normally in the GFP-SH2-Bβ (R555E) cell line in response to NGF treatment, yet still displays an inability to shift FKHR from the nucleus to the cytoplasm in response to NGF suggesting that SH2-Bβ acts at least in part, downstream of Akt phosphorylation.

Consistent with FKHR being redistributed to the nucleus where it is thought to be proapoptotic by virtue of stimulating the transcription of proapoptotic proteins, co-expression of FKHR and SH2-Bβ(R555E) appeared to be deleterious to the cells. In contrast to GFP and GFP-SH2-Bβ for which most of the cells co-expressed FKHR and either GFP or GFP-SH2-Bβ, significantly fewer cells could be found co-expressing FKHR and SH2-Bβ (R555E) in either PC12 cells or 293T cells. For example, in 293T cells deprived of serum overnight, 86% of cells expressing SH2-Bβ also expressed FKHR whereas only 16% of cells expressing SH2-Bβ (R555E) also expressed FKHR. Also consistent with nuclear FKHR being deleterious to the cell, when PC12 cells were transfected with cDNA encoding FKHR lacking its three AKT consensus phosphorylation sites, essentially no PC12 cells were found to express this FKHR mutant after 24 h (data not shown).

Discussion

The results presented in this study provide strong evidence that at least one mechanism by which the putative adapter protein SH2-Bβ contributes to the ability of NGF to promote survival of sympathetic neurons is to enhance the ability of NGF to activate Akt. Using the well-studied PC12 cell as a model system, we showed that NGF-induced activation of Akt is both enhanced and/or prolonged in cells overexpressing wild-type SH2-Bβ. This is most likely a consequence of the increased NGF-induced phosphorylation of Akt, since it correlates in magnitude and time course with the increased NGF-induced phosphorylation of Ser 473. Phosphorylation of Ser 473 and Thr 308 have both been shown to be obligatory for maximal activation of Akt (146). This increased activity of Akt is most likely responsible for the similarly enhanced and prolonged NGF-induced phosphorylation of the Akt effector proteins, GSK-3, FKHRL1 and FKHR. The simplest explanation for this activation of Akt is that it is a consequence of the reported ability of SH2-Bβ to enhance and prolong NGF-induced tyrosyl phosphorylation of TrkA (96). Based upon what is known about how Akt is activated by receptor tyrosine kinases (102, 143-146), we would predict that the more highly phosphorylated TrkA would recruit more PI3 kinase, which in turn would stimulate the recruitment of more Akt to the plasma membrane, which would result in more molecules of Akt being phosphorylated on Ser473 and Thr 308 by phosphoinositide-dependent kinases. The NGF-induced activation of Akt (and phosphorylation of its downstream effectors GSK-3, FKHRL1, and FKHR) reported here, like the previously reported activation of TrkA, is enhanced by overexpression of SH2-B, but is not affected by ectopic expression of a form of SH2-B expected to act as a dominant negative, such as SH2-Bβ(R555E) (33) (in the case of Akt) or an N-terminally truncated form of SH2-B (in the case of TrkA) (96). It is also possible that SH2-Bβ enhances and

prolongs NGF activation of Akt by acting at a point downstream of TrkA, for instance by blocking the dephosphorylation of Akt.

Enhanced NGF-induced phosphorylation of Akt substrates presumably contributes to the ability of SH2-B to protect against axonal degeneration and/or neuron cell death. GSK-3, which exhibits increased NGF-induced phosphorylation in PC12 cells stably expressing SH2-Bβ, is inhibited upon phosphorylation by Akt (117) and inhibition of GSK-3 has been reported to reduce neuronal death caused by inhibition of Akt (169). One mechanism proposed by which inhibition of GSK-3 contributes to neuronal survival is by stabilization of β-catenin (170). Downregulation of GSK-3 as a consequence of SH2-Bβ enhanced phosphorylation would also be expected to prevent neurite retraction. It seems likely that SH2-Bβ enhancement of NGF-induced phosphorylation and consequent inactivation of other pro-apoptotic, Akt substrates, such as the Bcl2 family member BAD, will also be found to contribute to the survival effects of SH2-Bβ.

The lack of a negative effect of SH2-Bβ(R555E) on Akt activity was somewhat surprising to us, given the ability of this mutant to completely block NGF-induced neuronal differentiation of PC12 cells (33) and our anecdotal observation that this mutant SH2-Bβ promotes cell death. This suggested to us that the ability of SH2-Bβ to enhance and prolong NGF-induced activation of Akt may not be the only mechanism by which SH2-Bβ contributes to NGF-induced differentiation and/or survival of PC12 cells and/or sympathetic neurons. The finding that SH2-Bβ(R555E) profoundly affects the subcellular distribution of FKHR, even in the absence of NGF, provides support for the hypothesis that SH2-Bβ affects more than just Akt-mediated phosphorylation of FKHR. Based upon the current dogma (118), activated Akt moves to the nucleus where it can phosphorylate members of the FKHR family on multiple Ser/Thr. The phosphorylated FKHR binds to 14-3-3 which facilitates its export from the nucleus and may facilitate retention in the cytoplasm. Recently, the

phosphorylation of two novel casein kinase 1 sites within FKHR, Ser 322 and Ser325, were shown to be required for FKHR to interact with the export machinery (171). Because FKHR members have been implicated in the regulation of a variety of apoptotic genes, including Fas ligand and cyclin dependent kinase inhibitor $p27^{KIP1}$ (172), inhibition of FKHR activity by keeping it out of the nucleus would promote cell survival. The current results suggest that SH2-B may participate in regulating the subcellular distribution of FKHR. One scenario is that SH2-B acts as a scaffolding protein that promotes the interaction of FKHR with 14-3-3. Another possibility is that SH2-B facilitates the transport of FKHR out of the nucleus. One can envision SH2-Bβ(R555E) acting as a dominant negative by binding to some but not all of the proteins necessary for FKHR interaction with 14-3-3 or for nuclear export of FKHR. The likelihood of this possibility is suggested by our recent finding (Chen and Carter-Su, submitted for publication) that SH2-Bβ itself cycles between the cytoplasm and the nucleus. Alternatively, SH2-Bβ might take one or more of the proteins required for these events to the wrong subcellular location or affect the ability of FKHR to be phosphorylated on the casein kinase I phosphorylation sites Ser322 and Ser325. Any of these scenarios could occur as a consequence of SH2-Bβ(R555E) not binding to TrkA (or other neurotrophic receptor tyrosine kinases) and/or not being phosphorylated on tyrosines, serines, or threonines as a consequence of not binding to activated TrkA (33, 68). However, the fact that overexpression of SH2-Bβ causes FKHR to shift to the cytoplasm in the absence of NGF suggests that modification of SH2-Bβ by TrkA is not necessary for SH2-Bβ to affect the subcellular distribution of FKHR.

In summary, using PC12 cells as a model system, we have provided substantial evidence that SH2-Bβ enhances and prolongs NGF activation of Akt and phosphorylation of its downstream effectors, including GSK-3α and β, FKHRL1, and FKHR. This action would be expected to contribute to the observed ability of overexpressed SH2-Bβ to enhance NGF-induced neuronal differentiation of

PC12 cells and survival of sympathetic neurons (33, 34). SH2-Bβ appears to have an additional effect on FKHR and potentially other members of the FKHR family, namely promoting or retaining FKHR in the cytoplasm, thereby preventing it from acting as a transcription factor. We are actively involved in determining the mechanisms by which SH2-Bβ and its mutants affect the subcellular localization of FKHR family members.

Acknowledgements

We thank B. Hawkins for assistance with the figures and the manuscript. We thank Drs. E. Tang and K. Guan for the gift of FKHR cDNA. This research was supported by NIH grant R01-DK54222 and The Juvenile Diabetes Research Foundation Center for the Study of Complications of Diabetes. TM was supported by a Predoctoral Traineeship in Cellular and Molecular Biology GM-07315 from the NIH. This work used the Core Facilities (Morphology and Image Analysis, and Cell and Molecular Biology Cores) of the Michigan Diabetes Research and Training Center (P60-DK20572).

This Chapter has been published in **Journal of Biological Chemistry** (2004) Vol279, No. 1, pgs. 133-141, under the title “SH2-B Is a Positive Regulator of Nerve Growth Factor-mediated Activation of the Akt/Forkhead Pathway in PC12 Cells” by Xiaohong Wang, Linyi Chen, Travis J. Maures, James Herrington, and Christin Carter-Su. Xiaohong Wang and Linyi Chen provided FIGs 2.1-2.5 while I provided FIGs 2.6-2.8. Xiaohong Wang, Linyi Chen, Travis J. Maures contributed equally to this work.

Chapter 3

SH2B1β (SH2-Bβ) Enhances Expression of a Subset of Nerve Growth Factor-Regulated Genes Important for Neuronal Differentiation Including Genes Encoding UPAR and MMP3/10

Abstract

Previous work showed that the adapter protein SH2B1 (SH2-B) binds to the activated form of the nerve growth factor (NGF) receptor TrkA and is critical for both NGF-dependent neurite outgrowth and maintenance. To identify SH2B1β regulated genes critical for neurite outgrowth, we performed microarray analysis of control PC12 cells and PC12 cells stably overexpressing SH2B1β (PC12-SH2B1β) or the dominant negative SH2B1β(R555E) (PC12-SH2B1β[R555E]). NGF-induced microarray expression of Plaur and Mmp10 genes was greatly enhanced in PC12-SH2B1β cells while NGF-induced Plaur and Mmp3 expression was substantially depressed in PC12-SH2B1β(R555E) cells. Plaur, Mmp3 and Mmp10 are among the 12 genes most highly upregulated after 6 h of NGF. Their protein products (uPAR, MMP3, MMP10) lie in the same pathway of extracellular matrix degradation; uPAR has been shown previously to be critical for NGF-induced neurite outgrowth. QT-PCR analysis revealed SH2B1β-enhancement of NGF-induction of all three genes and the suppression of NGF-induction of all three when endogenous SH2B1 was reduced using shRNA against SH2B1 and in PC12-SH2B1β(R555E) cells. NGF-induced levels of uPAR and MMP3/10 and neurite outgrowth through Matrigel (MMP3-dependent) were also increased in PC12-SH2B1β cells. These results suggest that SH2B1β stimulates NGF-induced neuronal differentiation at least in part by

enhancing expression of a specific subset of NGF-sensitive genes, including Plaur, Mmp3 and/or Mmp10, required for neurite outgrowth.

Introduction

Nerve growth factor (NGF) plays a critical role in the development of the peripheral nervous system. This is especially true for sympathetic neurons, which are over-produced during development and are believed to compete for the limited quantities of target-derived NGF for their survival; only the select neurons that successfully innervate NGF-producing target organs persist (9, 173, 174). Sympathetic neuron requirement for NGF for survival has been well documented both in vitro and in vivo [reviewed in (175, 176)]. However, due to the absolute NGF dependence of sympathetic neurons for survival, it has been difficult to assess the necessity for NGF on other aspects of sympathetic neuron development and function, such as axonal growth and target innervation. The use of Bax knockout mice circumvented this problem. Deletion of Bax, a pro-apoptotic transcription factor, dramatically reduces trophic-factor deprivation-induced apoptosis of both sensory and sympathetic neurons (177). The sensory and sympathetic neurons that die off in mice lacking NGF (NGF-/-) and the NGF receptor TrkA (TrkA-/-) (11, 178) are able to persist when those mice are crossed with the Bax-/- mice (12). In the double knockout mice, axons from both sympathetic and sensory neurons appear unable to reach their targets, resulting in impaired target innervation (6, 12, 13). Although experiments using these mice implicate NGF as an essential signal required for normal axonal growth of and peripheral target innervation by sympathetic and sensory neurons in vivo, the actual mechanisms by which NGF elicits these effects are only beginning to be understood. Most of what we know about NGF-induced neuronal differentiation and NGF signal transduction events has come from the PC12 cell model. PC12 cells, derived from a rat pheochromocytoma, can be induced to differentiate into sympathetic neuron-like cells by NGF (16, 179). Upon exposure to NGF, these cells first cease to proliferate. They then exhibit somatic hypertrophy, acquire neurites and express neuronal specific genes. The neurite outgrowths are

capable of forming typical looking synapses with primary neurons from rat cortex (18). As has been documented for sympathetic and sensory neurons, once PC12 cells differentiate into a neuronal phenotype, they depend on NGF for survival [for review, see (180)].

NGF initiation of neuronal differentiation starts with NGF binding to its receptor tyrosine kinase TrkA and activating TrkA. The activated TrkA autophosphorylates and the phosphorylated tyrosines in the TrkA cytoplasmic domain bind a number of different signaling molecules and initiate a variety of downstream signaling pathways implicated in neuronal differentiation (181, 182). Binding proteins include Shc, phospholipase C-γ (PLC-γ), fibroblast growth receptor substrate 2 (Frs2, SNT), and CHK, a homolog of the cytoplasmic tyrosine kinase CSK (control of Src kinase). Binding of these and presumably other TrkA binding proteins lead to the activation of PLC-γ/Ca2+; NFκB; phosphatidylinositol-3-kinase (PI3K)/Akt; Ras/Raf/MEK/ extracellular regulated kinases (ERKs) 1 and 2; JNK; p38; and atypical protein kinase C pathways [reviewed in (19), (20)].

We and others identified the putative adapter/scaffold protein SH2B1 as one of the signaling proteins that bind to the NGF-activated form of TrkA (33, 34). NGF promotes the rapid association of SH2B1 with TrkA and subsequent phosphorylation of SH2B1 on tyrosines as well as serines/threonines (33, 34, 68). SH2B1 belongs to a family of adapter proteins that also includes APS (SH2B2) and Lnk (SH2B3) (43-45). SH2B1 family members contain a pleckstrin homology (PH) domain, at least one dimerization domain and a C-terminal Src homology (SH2) domain [reviewed in (183)]; it is the SH2 domain that binds to TrkA. The four known SH2B1 splice variants, α, β, γ, and δ, differ only in their C-termini starting just past the SH2 domain (33, 37, 46), suggesting that all isoforms would be recruited to TrkA and would share many but perhaps not all, responses. SH2B1 has been implicated in NGF-induced neurite outgrowth of PC12 cells (33, 34). Thus, overexpression of SH2B1α or SH2B1β enhances NGF-induced neurite outgrowth in PC12 cells. In contrast, mutating a critical

arginine (SH2B1β[R555E]) within the SH2 domain of SH2B1β that prevents SH2B1β from binding to and being phosphorylated by TrkA, blocks NGF-induced neurite outgrowth of PC12 cells (33, 68). SH2B1 has also been implicated in the NGF-dependent maintenance of explants of sympathetic cervical ganglia (34). Dissociated primary sympathetic neurons growing in NGF-containing medium exhibit a reduced rate of survival when anti-SH2B1 antibodies are introduced by trituration. Similarly, axonal processes are nearly eliminated when an SH2B1 mutant that blocks SH2B1-mediated signaling is introduced within explants of sympathetic ganglia grown in the presence of NGF. In contrast, NGF-treated neurons into which wild-type SH2B1 is introduced thrive and have elaborate, long-branching axonal processes.

The mechanism by which SH2B1 enhances neurite outgrowth is unknown. Qian and Ginty (96) suggest that SH2B1 potentiates NGF-induction of autophosphorylation of TrkA, assessed using antibody to phosphotyrosine 490 in TrkA, the binding site for Shc. Such a generalized stimulation of TrkA activity by SH2B1 might be expected to enhance the expression of all NGF-sensitive genes. However, SH2B1 potentiation of NGF-induction of TrkA activity cannot explain the ability of the dominant negative SH2B1β(R555E) to block NGF-induced neurite outgrowth, since PC12-SH2B1β(R555E) cells did not exhibit decreased TrkA autophosphorylation in general (33) or on tyrosine 490 (Maures T and Carter-Su, C, unpublished observation) in response to NGF. The inability of SH2B1β(R555E) to inhibit NGF-induced tyrosyl phosphorylation of TrkA, phospholipase Cγ or Shc or activation of ERKs 1 and 2 led us to hypothesize that SH2B1β may initiate a previously unknown NGF signaling pathway (33). SH2B1β contains both a nuclear export (79) and nuclear localization signal (Maures, T, Chen, L and Carter-Su, C, manuscript in preparation). Experiments using truncated and mutated forms of SH2B1β, as well as the inhibitor of nuclear export leptomycin B, revealed that both ectopically expressed and endogenous SH2B1β shuttle between the cytoplasm/plasma membrane and the nucleus (79).

Unlike wild-type SH2B1β, SH2B1β mutants with a defective nuclear export (79) or import (Maures, T, Chen, L and Carter-Su, C, manuscript in preparation) signal are unable to enhance NGF-induced neurite outgrowth. Together, these findings led us to hypothesize that while SH2B1β might have a modest stimulatory effect on multiple NGF-sensitive genes, there would exist a specific subset of genes important for neurite outgrowth that were particularly sensitive to SH2B1β.

To determine whether SH2B1β regulates all or a subset of NGF-responsive genes early in the NGF-induced neuronal differentiation program, and at the same time identify SH2B1-regulated genes important for neurite outgrowth, we performed microarray analysis on cRNA prepared from NGF-treated PC12 cells stably expressing SH2B1β, SH2B1β(R555E), or SH2B1β vector. We identified both a subset of NGF-responsive genes whose NGF-regulated expression is enhanced substantially further by SH2B1β and/or substantially dampened by SH2B1β(R555E), as well as a subset of genes whose expression is not altered by SH2B1β or SH2B1β(R555E). We confirmed the NGF and SH2B1β regulation of a subset of these SH2B1β regulated genes using QT-PCR. We further characterized SH2B1β regulation of the proteins encoded by three of the genes most highly up-regulated by NGF and whose NGF up-regulation appeared to depend upon SH2B1β: Plaur, Mmp3 and Mmp10, encoding urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase (MMP) 3 (stromelysin-1; transin-1) and MMP10 (stromelysin-2; transin-2), respectively. These proteins were of particular interest because uPAR has been shown previously to be required for NGF-induced neuronal differentiation and MMP3 and MMP10 lie in the same extracellular matrix degradation pathway as uPAR. Consistent with MMP3 being important for neurite outgrowth and penetration through an extracellular matrix, we showed that SH2B1β enhances neurite extension through Matrigel. Finally, we confirmed a critical role for endogenous SH2B1 in the expression of these proteins using shRNA to SH2B1,

which was found to suppress NGF-induced expression of Plaur, Mmp3 and Mmp10.

Materials and Methods

Antibodies and reagents. Polyclonal antibody to rat SH2B1 (αSH2B1), kind gift of Dr. Liangyou Rui (University of Michigan), was raised against an SH2B1β glutathione S-transferase fusion protein (93) and used at a dilution of 1:1000 for western blotting. Monoclonal antibody to neuronal β-tubulin (TUJ1) was from Covance (MMS-435P) and used at a dilution of 1:1000 for immunostaining of neurites. Antibody to α-tubulin (Cell Signaling) was used at a dilution of 1:1000 for western blotting. Polyclonal anti-MMP3 antibody (αMMP3) was from Chemicon (Cat#AB19150) and used at 1:1000 for western blotting. A polyclonal antibody against the ligand-binding NH(2)-terminal domain of rat uPAR was used for western blots at a dilution 1:500 as previously described (184). Anti-mouse uPAR monoclonal antibody used for immunostaining (at a dilution of 1:1000) was from R&D System (Cat#AF534). Alexa Fluor 555-conjugated anti-mouse IgG used for immunocytochemistry and Alexa Fluor 680-conjugated anti-rabbit IgG used for western blotting came from Invitrogen. NGF, rat-tail collagen I and growth factor-reduced Matrigel were purchased from BD Bioscience. Purified human MMP3 was from Triple Points Biologicals (Cat#H-MMP-3, 0.1ug/ul), fluorescein was from BioRad and Syber green I was from Sigma. TaqMan RT-PCR kit was purchased from Applied Biosystem (Roche) (Cat#N808-0234).

Stable cell lines and cell culture. The stock of PC12 cells was purchased from American Type Culture Collection. PC12 cells were plated on collagen-coated plates (0.1 mg/ml rat tail collagen in 0.02 N acetic acid) and grown at 37°C in 10% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated horse serum (ICN), 5% fetal bovine serum (Invitrogen), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen). PC12 cells stably overexpressing GFP (control PC12 cells), GFP-SH2B1β (PC12-SH2B1β cells), or GFP-SH2B1β(R555E) (PC12-SH2B1β[R555E]

cells) were described previously (79). Experimental plates were incubated in serum-free medium overnight and then treated with vehicle or 100 ng/ml NGF for the indicated times.

RNA preparation and microarray analysis. Total RNA was isolated from control and NGF-treated cells using Stat60 (Tel-Test, Inc.) and Rneasy spin columns (Qiagen) according to the manufacturers' instructions. RNA from five experiments, each performed in duplicate, was prepared and the quality of RNA checked by optical density and agarose gels. To assess further the quality of the RNA and confirm that the cells responded to NGF, QT-PCR was used to monitor the expression level of some known NGF-responsive genes. Based on these results, RNA from duplicates of two independent experiments was combined in equal proportions for microarray analysis. Increasingly, data sets in the Gene Expression Omnibus Database use pooled RNA samples for microarray analyses; the advantage of pooled RNA samples has been documented (185, 186). Preparation of cRNA from equal amounts of pooled RNA per cell line and its hybridization to rat genome RAE233A oligonucleotide arrays (Affymetrix) was performed by the Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center. The averaged NGF responsiveness (+NGF/-NGF) was used in Tables 1,2 and 4 for genes represented by more than one probe set. Probe sets with raw data less than 100 in all six samples were not included in Tables 1-4 to minimize false positive results. For calculating NGF responsiveness, values of raw data that are smaller than 50 are regarded as 50.

QT-PCR. Gene expression of Plaur, Mmp3, Mmp10, Stc1, Ca2 and Glrx1 was determined by QT-PCR using SYBR green I and the iCycler system with iCycler iQ Real Time Detection System software (BioRad Laboratories). Primer sequences were designed using PrimerExpress software and are listed in Table 5. Amplicons generated from each primer pair were 50-52bp. Loading of each sample was normalized with fluorescein. All readings were normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of GAPDH expression were not different in control PC12, PC12-SH2B1β

and PC12-SH2B1β[R555E] cells (FIGs. 3.3 and 3.4) nor in shControl versus PC12-shSH2B cells (FIG. 3.9B).

Cell lysis and immunoblotting. Cells were washed three times with chilled PBS (10 mM sodium phosphate, 137 mM NaCl, pH 7.4) containing 1 mM Na_3VO_4, solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue), and boiled for 10 min before centrifugation at 13,200 rpm for 1 min. Equal amounts of the solubilized proteins were separated on SDS-PAGE gels, transferred to PVDF (FIG. 3.6A) or nitrocellulose (FIG. 3.9A) membranes, immunoblotted with the indicated antibody, and detected using Alexa Fluor 680-conjugated anti-rabbit IgG and an Odyssey Infrared Imaging System (LI-COR Biosciences).

Analysis of uPAR distribution. PC12 cells were plated onto Matrigel coated coverslips, deprived of serum and incubated for the indicated time with 100 ng/ml NGF. Cells were fixed with 4% paraformaldehyde, incubated with uPAR monoclonal antibody, followed by Alexa Fluor 555-conjugated secondary antibody. Analysis of uPAR distribution was performed with an Olympus FluoView 500 laser scanning confocal microscope utilizing a 60X oil-immersion objective and FluoView version 5.0 software. Alexa Fluor 555 fluorescence was excited with a Green Helium Neon (HeNe) laser at 543 nm and emission was measured through a 560 nm long pass filter (560 and above). GFP fluorescence was excited with an Argon (Ar) laser at 488 nm and emission was measured through a 505-525 nm filter.

Zymogram gel analysis. PC12 cells were incubated overnight in DMEM containing 0.1% chicken ovalbumin and then treated with 100 ng/ml NGF as indicated. Proteins in conditioned medium were concentrated 3-4 fold using Centricon 30 concentrator (Millipore) and separated on a 0.1% casein-containing, pre-stained, Tris glycine gel (Invitrogen, Inc.) under non-reducing conditions. The gel was washed three times with renaturation buffer (2.5% Triton X-100) to remove SDS and renature the MMPs and then developed overnight at 37°C in

developing buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35) to visualize regions in the gel which contain active MMP.

Neurite outgrowth through Matrigel. Neurite outgrowth through Matrigel was assessed using a modified method of Nordstrom *et al*. (123). Briefly, 50 μl of 1:3 diluted Growth Factor Reduced, phenol red-free Matrigel (BD Biosciences) was added to the *cis* plane of a 24-well transwell membrane (6.5 mm^2, 5 μm pore size) and allowed to solidify at 37°C for 30 min. The *trans* plane of the transwell membrane was coated with 1:10 diluted Growth Factor Reduced Matrigel overnight at room temperature. Cells were plated onto the *cis* plane of the transwell membrane at a density of 50,000 cells/well in phenol red-free DMEM containing 0.1% chicken ovalbumin. NGF (100 ng/ml) was added to the outer chamber. After 4 days, cells were fixed and immunostained for neuronal β-tubulin (TUJ1) followed by Alexa Fluor 555-conjugated secondary antibody. Neurites on the *trans* plane of the membrane were visualized by fluorescence microscopy (Nikon Eclipse TE2000) and counted.

Silencing of SH2B1 gene. SH2B1 siRNA vector was constructed by inserting an oligonucleotide containing the SH2B1 sequence (5'-CATCTGTGGTTCCAGTCCA-3') corresponding to nucleotides 1771-1789 of *rat SH2B1* (GenBank™ accession number AF047577) into the pSuper retro vector containing the puromycin resistance gene (pSuper retro puro) (OligoEngine). A pSuper vector containing a non-targeting siRNA with a low sequence similarity to known genes was used as a control. The SH2B1 and control siRNA vectors were transfected into subconfluent PC12 cells using a BioRad Gene Pulser Xcell electroporator (400V, 500μF, 0.4 cm cuvette). After 14h, cells were washed with PBS and fresh growth medium was added. Twenty-four hours later, the medium was changed to a selection medium containing 5 μg/ml puromycin and selected for pSuper positive PC12 cells for 30 days. The efficacy of the SH2B1 RNAi was assessed using QT-PCR on RNA from the puromycin resistant PC12 lines (data not shown).

Results

Identification of a subset of NGF-responsive genes that are regulated by SH2B1β. To determine whether SH2B1β regulates the expression of all or a subset of NGF-sensitive genes in cells and at the same time identify SH2B1-regulated genes important for neurite outgrowth, we performed a global analysis of gene expression in pooled PC12 cells stably expressing GFP (control cells), GFP tagged wild-type SH2B1β (PC12-SH2B1β cells) or GFP tagged dominant negative SH2B1β(R555E) (PC12- SH2B1β[R555E] cells) treated with or without 100 ng/ml NGF for 6 h. Microarray analysis of control PC12 cells using the rat Affymetrix gene chip RAE223A that detects ~15,900 known genes and ESTs identified 511 genes and ESTs whose expression at least doubled in response to NGF (FIG. 3.1, Supplemental Table S1). Expression of 79 known genes more than tripled (Table 1). Microarray analysis of control PC12 cells identified a similar number (513) of genes whose expression was decreased by more than 50% following NGF treatment (FIG. 3.1, Supplemental Table S2). Together, these microarray studies indicate that NGF rapidly regulates (both positively and negatively) the expression of ~1000 genes/ESTs, many more than previously recognized. Among the 511 genes/ESTs whose expression was at least doubled by NGF in control cells, 34 of them showed a further doubling in NGF-induction in PC12-SH2B1β cells (FIG. 1, Table 2) while 153 of them

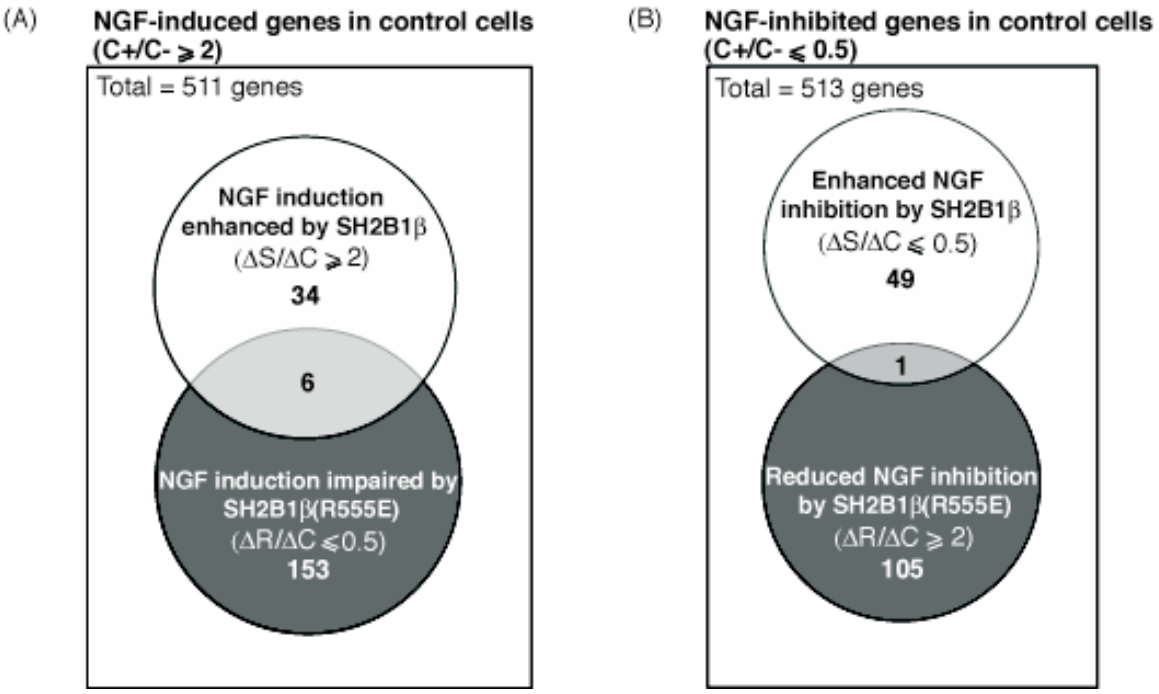


FIG. 3.1. SH2B1β enhances a subset of NGF-responsive genes.

(A) The expression of 511 genes and ESTs (588 probe sets) was at least doubled by treatment of control PC12 cells with NGF (6h, 100 ng/ml) (C+/C- ≥ 2). Among the 511 genes and ESTs, the NGF-induced expression of 34 genes and ESTs was enhanced by at least 100% by overexpression of SH2B1β, represented by the white circle (C+/C- ≥ 2 and ΔS /ΔC ≥ 2 where ΔS = S+/S- and ΔC = C+/C-). Among the 511 genes and ESTs, the NGF-induced expression of 153 genes was reduced by 50% or more in cells overexpressing SH2B1β (R555E), represented by the dark grey circle (C+/C- ≥ 2 and ΔR/ΔC ≤ 0.5 where ΔR = R+/R- and ΔC = C+/C-). There were only 2 genes and 4 ESTs that matched both criteria, represented by the overlapping area between the white and dark grey circles (C+/C- ≥ 2, ΔS/Δ C ≥ 2, ΔR/ΔC ≤ 0.5). (B) The expression of 513 genes and ESTs (507 probe sets) was reduced by at least 50% by treatment with NGF (6h, 100 ng/ml) (C+/C- ≤ 0.5). Among the 513 genes and ESTs, the expression of 49 genes and ESTs was further reduced by over-expression of SH2B1β, represented by the white circle (C+/C- ≤ 0.5 and ΔS/ΔC ≤ 0.5). The NGF-induced inhibition of 105 genes and ESTs was, in addition, dampened by overexpression of SH2B1β(R555E), represented by the dark grey circle (C+/C- ≤ 0.5 and ΔR/ΔC ≥ 2). There was only 1 EST that matched both criteria, represented by the overlapping area between the white and dark grey circles (C+/C- ≤ 0.5, ΔS/ΔC ≤ 0.5, ΔR/ΔC ≥ 2). C-, S- and R-: gene expression levels in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells, respectively, in the absence of NGF. C+, S+ and R+: gene expression levels in NGF-treated control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells, respectively. ΔC=C+/C-; ΔS=S+/S-; ΔR=R+/R-.

Table 3.1. NGF induces increases in gene expression in PC12 cells.

Control PC12 cells were incubated for 0 or 6 h with 100 ng/ml NGF and gene expression profiles were assessed as described in MATERIALS AND METHODS. Gene symbols, gene titles and unigene numbers are listed for the 77 known genes exhibiting NGF-induced gene expression of at least 3 times basal (no NGF) in control PC12 cells (C+/C- ≥ 3), arranged in descending order based on the magnitude of the NGF induction of expression. The 74 ESTs that met this criterion are included in Supplement Table S1. C-, C+: gene expression levels in untreated and NGF-treated control PC12 cells, respectively. "a": Genes represented by more than one probe set; values listed are the average of the replicates.

Gene symb	Gene Title	Unigene	C+/C-
Mmp3	matrix metalloproteinase 3	Rn.32086	114.81
Crh	corticotropin releasing hormone	Rn.10349	56.80
Pai1	serine (or cysteine) proteinase inhibitor, member 1	Rn.29367	46.58
Mmp10	matrix metalloproteinase 10	Rn.9946	38.02
Mmp13	matrix metalloproteinase 13	Rn.10997	22.90
Egr1	Early growth response 1	Rn.9096	21.60
Ca2(a)	carbonic anhydrase 2	Rn.26083	14.81
Scn6a	sodium channel, voltage-gated, type 6, alpha poly p.	Rn.54541	12.32
Fosl1	Fos-like antigen 1	Rn.11306	10.22
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78
Vrl1	vanilloid receptor-like protein 1	Rn.44866	7.73
Vgf	VGF nerve growth factor inducible	Rn.9704	7.15
Krt1-18	keratin complex 1, acidic, gene 18	Rn.11104	6.78
Ret(a)	Ret proto-oncogene	Rn.44178	6.66
Dusp6	dual specificity phosphatase 6	Rn.4313	6.61
Ania4	activity and neurotransmitter-induced early gene 4	Rn.40517	6.41
Klf5	Kruppel-like factor 5 (intestinal)	Rn.17478	5.50
Bmp1	bone morphogenetic protein 1 (procollagen C-proetinase)	Rn.87080	5.43
Giot2	gonadotropin inducible ovarian transcription factor 2	Rn.77686	5.19

Nid67	putative small membrane protein NID67	Rn.8865	5.18
Glrx1(a)	glutaredoxin 1 (thioltransferase)	Rn.1484	4.94
Ifrd1	interferon-related developmental regulator 1	Rn.3723	4.90
LOC286921	aldose reductase-like protein	Rn.23676	4.83
Tage4	tumor-associated glycoprotein pE4	Rn.10677	4.83
Tjp2	tight junction protein 2	Rn.10965	4.70
Gpr37	G protein-coupled receptor 37(endothelin receptor type B-like)	Rn.28035	4.69
Tub	Tubby (mouse) homolog	Rn.30017	4.66
Tfpi	tissue factor pathway inhibitor	Rn.15795	4.56
Prkaa2	AMP-activated protein kinase	Rn.64583	4.48
Pcscl	peroxisomal Ca-dependent solute carrier-like protein	Rn.17644	4.47
Cga	glycoprotein hormones, alpha subunit	Rn.10589	4.22
Ctsk	cathepsin K	Rn.5598	4.20
Ager	advanced glycosylation end product-specific receptor	Rn.9829	4.10
Scya2	small inducible cytokine A2	Rn.4772	4.10
Fgf15	fibroblast growth factor 15	Rn.81230	4.04
Arc	activity regulated cytoskeletal-associated protein	Rn.10086	3.98
Lnk	linker of T-cell receptor pathways	Rn.11228	3.98
Bat3(a)	HLA-B-associated transcript 3	Rn.22304	3.97
Itga1	integrin, alpha 1	Rn.91044	3.87
Hes1	hairy and enhancer of split 1 (Drosophila)	Rn.19727	3.87
Ptafr	platelet-activating factor receptor	Rn.10137	3.80
Lgals3	lectin, galactose binding, soluble 3	Rn.764	3.79
Rgs5	regulator of G-protein signaling 5	Rn.1150	3.78
Glrx1	glutaredoxin 1 (thioltransferase)	Rn.1484	3.77
Synd1	Syndecan	Rn.11176	3.74
S100a10	S-100 related protein, clone 42C	Rn.4083	3.69

Ak4	adenylate kinase 4	Rn.44288	3.68
Scamp5	secretory carrier membrane protein 5	Rn.24420	3.66
Rdc1	chemokine orphan receptor 1	Rn.12959	3.65
Bdnf(a)	Brain derived neurothrophic factor	Rn.11266	3.59
Filip	filamin-interacting protein L-Filip	Rn.44931	3.59
Cdkn1a	cyclin-dependent kinase inhibitor 1A	Rn.10089	3.57
Ptgs1	prostaglandin-endoperoxide synthase 1	Rn.44404	3.51
Hpx	hemopexin	Rn.2380	3.50
Ecel1	endothelin converting enzyme-like 1	Rn.45803	3.47
Hk2	Hexokinase 2	Rn.91375	3.44
Clcn2	chloride channel 2	Rn.11073	3.39
Upb1	ureidopropionase, beta	Rn.11110	3.38
Snk	serum-inducible kinase	Rn.12100	3.38
Nr2f6	nuclear receptor subfamily 2, group F, member 6	Rn.25840	3.35
Junb	jun B proto-oncogene	Rn.15806	3.32
Prss11	protease, serine, 11	Rn.2782	3.32
Adcyap1r1	adenylate cyclase activating polypeptide 1 receptor 1	Rn.88408	3.32
Lasp1	LIM and SH3 protein 1	Rn.69815	3.30
Ehd4	pincher	Rn.7379	3.29
Msg1	melanocyte-specific gene 1 protein	Rn.8163	3.26
Gls	glutaminase	Rn.5762	3.23
Axcam	axonal-associated cell adhesion molecule	Rn.10117	3.17
Dcamkl1	double cortin and calcium/calmodulin-dependent protein kinase-like 1	Rn.80575	3.16
Stc1	stanniocalcin 1	Rn.10647	3.12
Cyp4a12	cytochrome P450, 4a12	Rn.10034	3.08
S100a4	S100 calcium-binding protein A4	Rn.504	3.06
LOC59314	CaM-KII inhibitory protein	Rn.42880	3.06

Slc6a2	solute carrier family 6(neurotransmitter transporter, noradrenalin),member 2	Rn.14577	3.05
Thrsp	Thyroid hormone responsive protein (spot14)	Rn.81140	3.04
Tceb3	transcription elongation factor B (SIII), polypeptide 3 (110kD)	Rn.37427	3.04
Anxa2	calpactin I heavy chain	Rn.28	3.04
Crmp4	Collapsin response mediator protein 4	Rn.8499	3.01

displayed a 50% or greater reduction in NGF-dependent expression in PC12-SH2B1β(R555E) cells (FIG. 3.1). Only two known genes (Plaur and Glrx1) and 4 ESTs fit both criteria (ie exhibit NGF-induction that was both enhanced by at least 100% in PC12-SH2B1β cells and reduced by 50% or more in PC12-SH2B1β(R555E) cells) (FIG. 3.1, Table 2).

Similarly, among the 513 genes/ESTs whose expression was down-regulated by NGF by at least 50%, 49 showed a susbstantially greater (at least a doubling of the NGF-inhibition seen in control cells) NGF-dependent reduction in expression in PC12-SH2B1β cells (FIG. 3.1, Table 4) while 105 exhibited a substantially diminished (by ≥ 50%) NGF-dependent decrease in expression in PC12-SH2B1β(R555E) cells (Supplemental Table S4). None of the known genes and only one EST fulfilled both of these criteria. The finding that some, but not all, of the NGF-responsive genes show enhanced responsiveness to NGF in SH2B1β-overexpressing cells and/or reduced responsiveness to NGF in SH2B1β(R555E)-expressing cells is consistent with SH2B1β enhancing the NGF-induced regulation of expression of a subset of NGF-responsive genes during neuronal differentiation.

Functional classification of NGF-responsive genes that are also regulated by SH2B1β. To obtain insight into the cellular processes that are regulated by SH2B1β, genes whose expression level was induced or inhibited by greater than a factor of two following treatment with NGF were grouped into Gene Ontology (GO) categories using DAVID (Data for Annotation, Visualization and Integrated Discovery) (187). For each of the three different cell lines, an average of 76% of input genes were categorized. As predicted given NGF's ability to promote neuronal differentiation, following stimulation of control PC12 cells with NGF for 6 h, the expression of genes associated with cell differentiation, intracellular signaling cascade, cell death, organogenesis, and morphogenesis was enhanced (FIG. 3.2A). Compared to control cells, PC12-SH2B1β cells had an increased number of NGF-induced genes overrepresented in all of the above-

mentioned categories whereas PC12-SH2B1β(R555E) cells had a decreased number. Also as predicted from NGF's ability to promote neuronal differentiation, genes whose expression in control PC12 cells was decreased by NGF by more than 50% were associated with cell cycle, cell proliferation, and cell migration (FIG. 3.2B). Overexpressing SH2B1β increased the number of NGF-induced genes overrepresented in all of the above-mentioned categories while overexpressing SH2B1β(R555E) reduced NGF-induced overrepresentation of genes in all 3 categories. In addition, genes in the category of cell differentiation were overrepresented in cells overexpressing SH2B1β and to a lesser extent in cells overexpressing SH2B1β(R555E). These findings are consistent with SH2B1β increasing NGF-induced neuronal differentiation at least in part by enhancing NGF-induced expression of a subset of genes whose products are implicated in morphogenesis, organogenesis, cell survival and intracellular signaling, and by increasing the degree of NGF-inhibition of expression of a subset of genes whose products are implicated in cell cycle progression, cell proliferation and cell migration. SH2B1β(R555E) appears to play a dominant negative role with regards to a subset of NGF regulated genes that affect morphogenesis, organogenesis, cell survival, intracellular signaling and cell differentiation, consistent with the substantially decreased ability of PC12-SH2B1β(R555E) cells to differentiate into a neuronal phenotype in response to NGF.

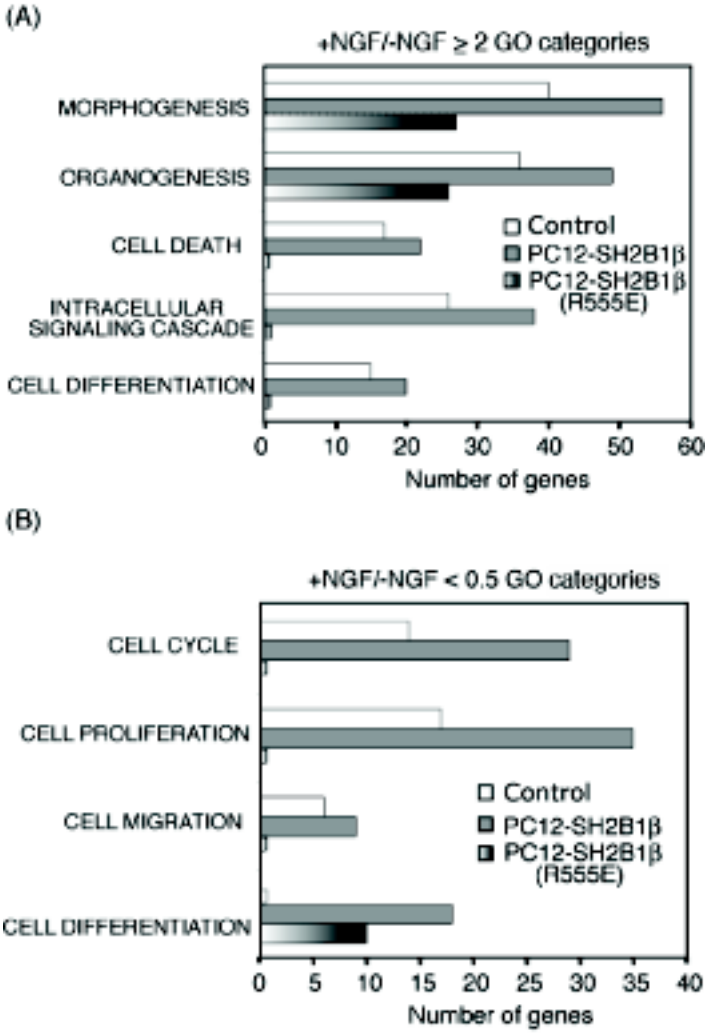


FIG. 3.2. Functional classification of NGF-responsive genes in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells.

Genes that were either up-regulated to at least twice the basal value by NGF (A) or down-regulated more than 50% by NGF (B) in control PC12, PC12-SH2B1β, and PC12-SH2B1β(R555E) cells were grouped into Gene Ontology categories. Overrepresented categories with $p<0.05$ are shown.

Novel SH2B1β-regulated genes likely to be involved in neuronal differentiation. Because PC12-SH2B1β and PC12-SH2B1β(R555E) cells display substantially enhanced and impaired neurite outgrowth, respectively, we reasoned that genes whose expression pattern is substantially enhanced following NGF treatment and whose NGF-enhancement is greatly increased in PC12-SH2B1β cells and/or substantially decreased in PC12-SH2B1β(R555E) cells would be prime candidates for further investigation. Seventeen known genes exhibited at least a doubling of expression in response to NGF in control cells and at least a further doubling in NGF-induced expression in PC12-SH2B1β cells (Table 2). Of these 17 genes, Mmp10 exhibited both the greatest NGF-induction of gene expression in control PC12 cells (+NGF/-NGF ~ 38) and the greatest further enhancement (~7 x) upon overexpression of SH2B1β. Mmp10 encodes matrix metalloproteinase 10 (MMP10), a proteinase involved in the degradation of the extracellular matrix [reviewed in (188)]. Among these same 17 genes, only Plaur and Glrx1 (highlighted in Table 2) also exhibited a significantly reduced (≥ 50% reduction) response to NGF in PC12-SH2B1β(R555E) cells compared to control cells. Plaur and Glrx1 encode urokinase plasminogen activator receptor (uPAR) and glutaredoxin 1, respectively. Plaur was of particular interest because of its being identified previously as a primary response gene for NGF critical for NGF-induced neurite outgrowth and biochemical differentiation of PC12 cells (121, 122). Glutaredoxin 1 has not been previously shown to be upregulated by NGF, much less by SH2B1, but was intriguing because it is found in neurons and modulates cellular redox status, important for cell survival against oxidative stress (189).

Table 3.2. NGF-responsive genes whose expression is enhanced by SH2B1β.

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or NGF for 6 h and gene expression profiles were assessed as described in MATERIALS AND METHODS. Gene symbols, gene titles and unigene numbers are listed for the 17 known genes for which NGF induces at least a doubling of expression in control PC12 cells (C+/C- ≥ 2) and at least a further doubling of NGF-induced expression in PC12-SH2B1β (ΔS/ΔC ≥ 2), arranged in order of decreasing ability of SH2B1β to enhance NGF-induced gene expression. An additional 18 ESTs (unlisted) met this criterion. The two genes highlighted in grey (and four ESTs not listed) additionally show a reduction in the magnitude of NGF-induction of expression in PC12-SH2B1β(R555E) cells to a level that is less than 50% that seen with control PC12 cells (ΔR/ΔC ≤ 0.5). C+, C-, S+, S-, R+, R-, ΔC, ΔS and ΔR are defined in FIG. 3.1. "a": Genes represented by more than one probe set; values listed are the average of the replicates. The sorting criterion of the table is designated in bold.

Gene Symbol	Gene Title	Unigene	C+/C-	S+/S-	R+/R-	ΔS/ΔC	ΔR/ΔC
Mmp10	matrix metalloproteinase 10	Rn.9946	271.78	45.24		7.15	1.19
Stc1	stanniocalcin 1	Rn.10647	3.12	20.87	2.05	6.69	0.66
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78	56.16	4.44	5.74	0.45
Scn6a	sodium channel, voltage-gated, type 6, alpha polypeptide	Rn.54541	12.32	65.40	23.36	5.31	1.90
Ca2(a)	carbonic anhydrase 2	Rn.26083	14.81	76.20	7.90	5.13	0.55
Fosl1	Fos-like antigen 1	Rn.11306	10.22	48.84	5.48	4.78	0.54
Arc	activity regulated cytoskeletal-associated protein	Rn.10086	3.98	14.44	6.85	3.62	1.72
Glrx1(a)	glutaredoxin 1 (thioltransferase)	Rn.1484	4.94	17.82	1.90	3.57	0.41

Dusp6	dual specificity phosphatase 6	Rn.4313	6.61	17.73	7.45	2.68	1.13
LOC286921	aldose reductase-like protein	Rn.23676	4.83	12.78	4.80	2.64	0.99
Ania4	activity and neurotransmitter-induced early gene protein 4 (ania-4)	Rn.40517	6.41	14.77	7.42	2.30	1.16
Egr1	Early growth response 1	Rn.9096	21.60	49.52	27.71	2.29	1.28
Kzf2	KRAB-zinc finger protein KZF-2	Rn.10664	2.02	4.62	2.03	2.29	1.01
Tage4	tumor-associated glycoprotein pE4	Rn.10677	4.83	10.70	4.29	2.22	0.89
Basp2	brain abundant, membrane attached signal protein 2	Rn.10928	2.23	4.64	1.40	2.08	0.63
Gprk5	G protein-coupled receptor kinase 5	Rn.6500	2.10	4.33	1.70	2.06	0.81
Bcl10	B-cell CLL/lymphoma 10	Rn.13007	2.20	4.51	2.03	2.05	0.92

In addition, there were 12 genes whose NGF-induced level of expression in PC12-SH2B1β cells did not meet our criterion of being substantially greater (at least a doubling) than in control cells but whose NGF-induced expression was substantially reduced (by ≥ 50%) in PC12-SH2B1β(R555E) cells ($1<(S+/S-)/(C+/C-)<2$, $(R+/R-)/(C+/C-)<0.5$) (Table 3). Among those genes, Mmp3 exhibited the greatest NGF-induction of expression in control PC12 cells (+NGF/-NGF ~ 115) as well as one of the greatest reductions (68%) in NGF-stimulated expression in PC12-SH2B1β(R555E) cells. Mmp3 was of interest because it encodes matrix metalloproteinase 3 (MMP3), a proteolytic enzyme present in growth cones of NGF-treated PC12 cells and previously implicated in neurite penetration through the extracellular matrix (123).

NGF inhibits the expression of almost as many genes as it enhances the expression of, suggesting that for neuronal differentiation to occur, reduction of gene expression is as important as enhancement of gene expression. Twelve known genes exhibited more than a 50% decrease in expression in response to NGF in control cells and a further 50% or greater decrease in NGF-responsiveness in PC12-SH2B1β cells (Table 4). Consistent with SH2B1β enhancing NGF-induced neuronal differentiation, a process associated with decreased cellular proliferation, this group of down-regulated genes includes two that encode for proteins involved in DNA replication: Pola2 (polymerase α subunit II) and Pold1 (DNA polymerase δ, catalytic subunit). Polymerase α subunit is required for DNA replication while DNA polymerase δ possesses 3' exonuclease activity that is important for repair during DNA replication (190). Expression of SH2B1β(R555E) dampened NGF-induced inhibition of expression of only one EST. Dampening of NGF-induced expression of no known genes (Suppl Table S4) achieved our predetermined criterion of ≥ 50%.

Table 3.3. NGF-responsive genes whose expression is inhibited by SH2B1β(R555E).

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or NGF for 6 h and gene expression profiles were assessed as described in MATERIALS AND METHODS. Gene symbols, gene titles and unigene numbers are listed for the 14 known genes whose NGF-induced expression was at least doubled in control cells (C+/C- ≥ 2), greater in PC12-SH2B1β cells than in control PC12 cells (ΔS/ΔC > 1) and reduced in PC12-SH2B1β(R555E) cells to a level that is less than 50% that seen with control PC12 cells (ΔR/ΔC ≤ 0.5), arranged in order of decreasing ability of SH2B1β(R555E) to impair NGF-induced gene expression (decreasing ΔR/ΔC). An additional 11 ESTs (unlisted) met this criterion. C+, C-, S+, S-, R+, R-, ΔC, ΔS and ΔR are defined in FIG. 3.1. The sorting criterion of the table is designated in bold.

Gene Sym	Gene Title	Unigene	C+/C-	S+/S-	R+/R-	ΔS/ΔC	ΔR/ΔC
Pai1	serine (or cysteine) proteinase inhibitor, member 1	Rn.29367	46.58	53.54	7.63	1.15	0.16
Vldlr	Very low density lipoprotein receptor	Rn.9975	2.25	2.35	0.65	1.04	0.29
Klf5	Kruppel-like factor 5 (intestinal)	Rn.17478	5.50	10.64	1.76	1.93	0.32
Mmp3	matrix metalloproteinase 3	Rn.32086	114.81	119.00	37.17	1.04	0.32
Pou2f1	POU domain, class 2, transcription factor 1	Rn.9992	2.24	3.38	0.78	1.51	0.35
Bcat1	branched chain aminotransferase 1, cytosolic	Rn.8273	2.73	3.72	1.00	1.36	0.37
Cryab	crystallin, alpha B	Rn.54554	2.28	2.32	0.92	1.02	0.40
Ecel1	endothelin converting enzyme-like 1	Rn.45803	3.47	5.57	1.43	1.61	0.41
Glrx1(a)	Glutaredoxin1 (thioltransferase)	Rn.1484	4.94	17.82	1.90	3.57	0.41

Msg1	melanocyte-specific gene 1 protein	Rn.8163	3.26	3.46	1.36	1.06	0.42
TA1	tumor-associated protein 1	Rn.32261	2.18	3.11	0.93	1.43	0.42
Nmrk	non MHC restricted killing associated	Rn.53994	2.24	2.44	1.00	1.09	0.45
Plaur	Plasminogen activator, urokinase receptor	Rn.82711	9.78	56.16	4.44	5.74	0.45
Sv2b	synaptic vesicle glycoprotein 2 b	Rn.9940	2.30	2.84	1.07	1.23	0.47

SH2B1β increases the expression of a subset of NGF-responsive genes. To systematically identify genes whose expression was substantially increased in response to NGF and whose NGF-induced expression was further substantially increased by overexpression of SH2B1β and/or substantially dampened by overexpression of SH2B1β(R555E), we applied a hierarchical clustering algorithm based on Pearson correlation coefficients to group genes based on the similarity of NGF-dependent expression patterns among the three stable cell lines (FIG. 3.3). Red represents increased mRNA levels, green denotes decreased mRNA levels, and color intensity represents the magnitude of the expression ratio. This analysis revealed, in addition to Plaur and Glrx1 which met our most stringent criteria of opposing two-fold or greater differences in NGF-regulated gene expression in PC12-SH2B1β and PC12-SH2B1β(R555E) cells versus control PC12 cells, several genes whose regulation followed the same pattern but did not quite meet the criteria of ≥ two-fold differences (in opposing directions) for both PC12-SH2B1β and PC12-SH2B1β(R555E) cells. NGF-regulated (known) genes that fit both of our criteria (greatly increased NGF induction in PC12-SH2B1β cells and significantly reduced NGF induction in PC12-SH2B1β[R555E] cells) include Fosl1 (encoding Fos-like antigen 1), Plaur (uPAR), Ca2 (carbonic anhydrase 2), Pai1 (serine or cysteine proteinase inhibitor, member 1), Stc1 (stanniolcalcin 1), Glrx1 (glutaredoxin 1) and Klf5 (kruppel-like factor 5). NGF-sensitive genes fitting just one of the criteria (increased NGF-responsiveness in PC12-SH2B1β cells or decreased responsiveness in PC12-SH2B1β[R555E] cells compared to control cells) included Mmp10 (increased NGF-responsiveness in PC12-SH2B1β cells) and Mmp3 and Crh (corticotropin releasing hormone) (decreased responsiveness in PC12-SH2B1β[R555E] cells). The NGF-induced expression (+NGF/-NGF) for genes representing each of these categories is listed in FIG. 3B. NGF-induced expression of Scn6a (sodium channel, voltage-gated, type 6, alpha polypeptide) and Egr1 (early gene response 1) was also greatly enhanced in PC12-SH2B1β

Table 3.4. Genes whose expression is suppressed by NGF and further suppressed by SH2B1β.

Control PC12, PC12-SH2B1β, or PC12-SH2B1β(R555E) cells were incubated with vehicle or 100 ng/ml NGF for 6 h and gene expression profiles were assessed as described in MATERIALS AND METHODS. Gene symbols, gene titles and unigene numbers are listed for the 12 known genes whose expression in control cells is both reduced by NGF treatment to less than 50% of basal levels (C+/C- ≤ 0.5) and whose NGF-induced reduction is further reduced by at least an additional 50% in PC12-SH2B1β cells (C+/C- ≤ 0.5, ΔS/ΔC ≤ 0.5), arranged in the order of decreasing SH2B1β-dependent inhibition of gene expression (decreasing ΔS/ΔC). 34 ESTs (not listed) fulfilled these criteria. C+, C-, S+, S-, R+, R-, ΔC, ΔS and ΔR are defined in FIG. 3.1. (a): Genes represented by more than one probe set; values are the average of the replicates. The sorting criterion of the table is designated in bold.

Gene Sym	Gene Title	Unigene	C+/C-	S+/S-	R+/R-	ΔS/ΔC	ΔR/ΔC
Pola2	DNA polymerase alpha subunit II	Rn.31773	0.44	0.10	0.30	0.23	0.69
Sord	Sorbitol dehydrogenase	Rn.11334	0.13	0.04	0.12	0.28	0.89
Gcgr	glucagon receptor	Rn.11225	0.24	0.07	0.30	0.31	1.23
Prkwnk4	protein kinase, lysine deficient 4	Rn.12652	0.49	0.17	0.82	0.34	1.66
Ga	liver mitochondrial glutaminase	Rn.10202	0.47	0.16	0.37	0.35	0.80
Hip1	huntingtin interacting protein 1	Rn.50506	0.47	0.17	0.59	0.35	1.24
Syt3	Synaptotagmin 3	Rn.48884	0.28	0.11	0.46	0.38	1.63
Rb2(a)	Retinoblastoma-related gene	Rn.11020	0.39	0.15	0.43	0.39	1.10
Cldn3	claudin 3	Rn.4513	0.48	0.19	0.48	0.40	1.01
U91561	pyridoxine 5-phosphate oxidase	Rn.6299	0.45	0.20	0.40	0.44	0.89
Rab3a	Ras-related small GTP	Rn.44409	0.37	0.17	0.46	0.46	1.26

	binding protein 3A						
Pold1	DNA polymerase delta, catalytic subunit	Rn.88690	0.27	0.13	0.32	0.48	1.21

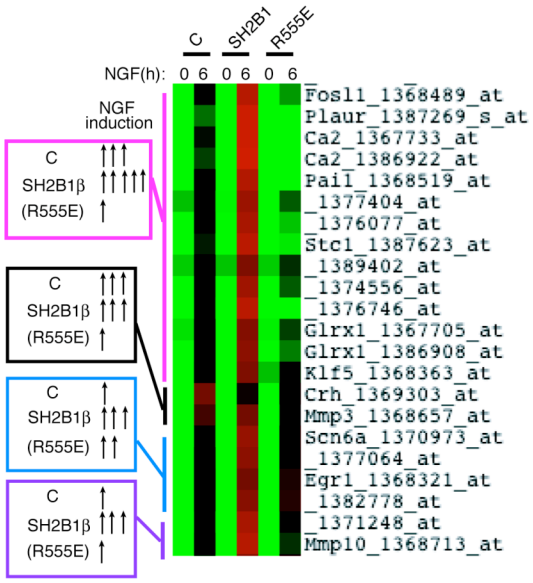
Table 3.5. Sequences of the QT-PCR primers used in this study.

Gene		Sequences
Mmp3	forward	5' TGAAGATGACAGGGAAGCTGG 3'
	reverse	5' GGCTTGTGCATCAGCTCCAT 3'
Mmp10	forward	5' GAAATGGTCACTGGGACCCTC 3'
	reverse	5' TGCGCAGCAACCAGGAATA 3'
GAPDH	forward	5' ATGACTCTACCCACGGCAAGTT 3'
	reverse	5' TCCCATTCTCAGCCTTGACTGT 3'
Plaur	forward	5' ACAGGACCATGAGCTACCGC 3'
	reverse	5' TCTCGGTGAGGCTGACGATC 3'
Stc1	forward	5' AACATGGCCAGCCTCTTCC 3'
	reverse	5' TGTCTGGGCACAGTGGTCTG 3'
Glrx1	forward	5' AGCATGGCTCAGGAGTTTGTG 3'
	reverse	5' CGACCACCTTTCCAGACTGAA 3'
Ca2	forward	5' TGGACATTGACACCGGGACT 3'
	reverse	5' GCAGAGGCTGTAGGGAAGGG 3'

cells compared to control cells but these genes were not explored further because their NGF-induced expression was also enhanced, albeit to a lesser extent, in PC12-SH2B1β(R555E) cells.

Quantitative real-time PCR confirms SH2B1 enhancement of expression of NGF-regulated genes identified using microarray analysis. To confirm NGF- and SH2B1β-regulation of expression of genes identified from the Affymetrix gene array analysis, we performed QT-PCR on representative genes from among the above genes showing by hierarchical clustering algorithm significantly increased (4 to 7 times greater) NGF-induced expression in PC12-SH2B1β cells vs control cells (Mmp10), substantially decreased (by 33-68%) NGF-induced expression in PC12-SH2B1β(R555E) cells vs control cells (Mmp3), or both (Plaur, Ca2, Stc1, Glrx1) (FIG. 3.3C). In general, the levels of NGF-induced gene expression in PC12-SH2B1β cells or PC12-SH2B1β(R555E) cells relative to control cells determined by QT-PCR paralleled those found by microarray analysis. Thus, the expression of all 6 genes was substantially increased in response to NGF in control PC12 cells. Also as predicted, compared to control cells, the NGF-stimulated response was substantially greater in PC12-SH2B1β cells for Plaur, Ca2, Stc1, Glrx1 and Mmp10 and lower in PC12-SH2B1β(R555E) cells for Plaur, Ca2, Glrx1, and Mmp3. In addition, NGF-induced expression was modestly greater in PC12-SH2B1β cells than in control cells for Mmp3 and modestly lower in PC12-SH2B1β(R555E) cells vs control cells for Mmp10. Thus, all 6 of the genes showed an increased NGF-induced expression when SH2B1β was overexpressed and 5 of the 6 genes showed decreased NGF-induced expression when the dominant negative SH2B1β(R555E) was expressed, consistent with these genes being regulated by SH2B1β and mediating at least in part SH2B1's effect on NGF-induced neuronal differentiation.

(A)



(B)

	C +NGF/-NGF	SH2B1β +NGF/-NGF	R555E +NGF/-NGF
Plaur	9.78	56.16	4.44
Ca2	17.62	92.12	8.41
Ca2	12.00	60.29	7.39
Stc1	3.12	20.87	2.05
Glrx1	6.10	22.88	1.83
Glrx1	3.77	12.75	1.97
Mmp3	114.80	119.00	37.1
Mmp10	38.02	271.78	45.24

FIG. 3.3A, B. NGF-responsive genes that are highly regulated by SH2B1β.
(A) NGF-induced gene expression profiles in control PC12 ("C"), PC12-SH2B1β ("SH2B1β"), and PC12-SH2B1β(R555E) ("R555E") cells were clustered and visualized using Eisen software (http://rana.lbl.gov/EisenSoftware.htm). Four different clusters of genes that show NGF-induced gene expression in all three cell types are shown. For each gene, black represents the average value of all six probe sets. The relative intensity of red represents the degree of increased mRNA levels and the relative intensity of green represents the degree of decreased mRNA levels. The number of arrows represents the relative levels of increase. (B) Microarray data for selected genes from each of the four clusters.

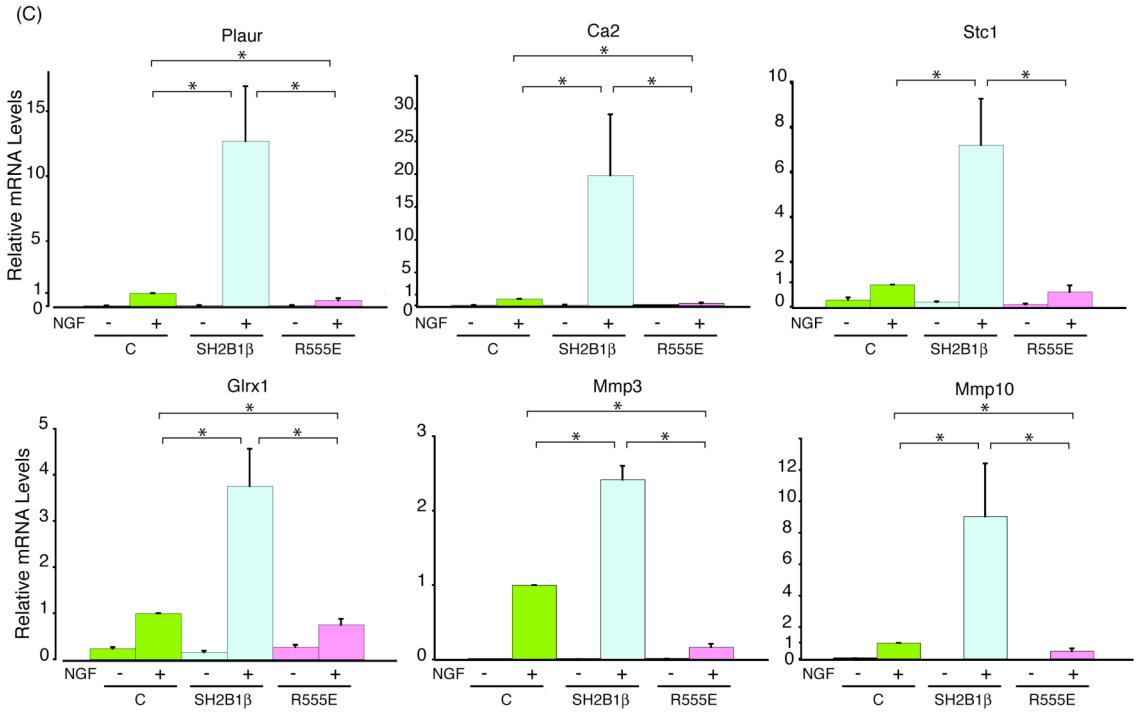


FIG. 3.3C. NGF-responsive genes that are highly regulated by SH2B1β.
(C) Gene expression levels for six of the genes shown in (A) were verified by QT-PCR, normalized first to levels of GAPDH gene expression and then to levels of gene expression seen in control PC12 cells treated with NGF. Means ± SEM from 3-4 experiments are shown. Asterisks represent statistically significant differences ($p<0.05$) using a one-tailed, paired Student T-test.

Time course of NGF-induced expression of SH2B1β regulated genes. To determine whether overexpression of SH2B1β increases overall NGF-induced expression of genes or simply shifts the time course of NGF-induced changes in expression, we used QT-PCR to compare the time course of NGF-inducement of expression of Stc1, Plaur, Mmp3, Mmp10, Ca2 and Glrx1 genes in control and PC12-SH2B1β cells (FIG. 3.4). For all genes tested, the time of onset of NGF-induced gene expression appeared similar in control and PC12-SH2B1β cells. Similarly, expression of all tested genes was elevated at multiple time points in PC12-SH2B1β compared to cells and the majority of the tested genes had their highest level of induction at 4 or 6h in both control and PC12-SH2B1β cells. Thus, for no gene could the difference in level of NGF induction in PC12-SH2B1β cells compared to control cells seen after 6 h of NGF be attributed to a different time course of NGF responsiveness. However, it is interesting to note that NGF-induced expression of both Mmp3 and Mmp10 was prolonged in PC12-SH2B1β cells compared to control cells. In contrast to control cells in which Mmp3 and Mmp10 expression declined to near basal values after 24h of NGF, Mmp3 and Mmp10 expression in PC12-SH2B1β cells remained elevated (Mmp3) or may have even been still rising (Mmp10) after 24 h. These data indicate that SH2B1β both enhances and prolongs NGF-induction of expression of a subset of NGF-sensitive genes.

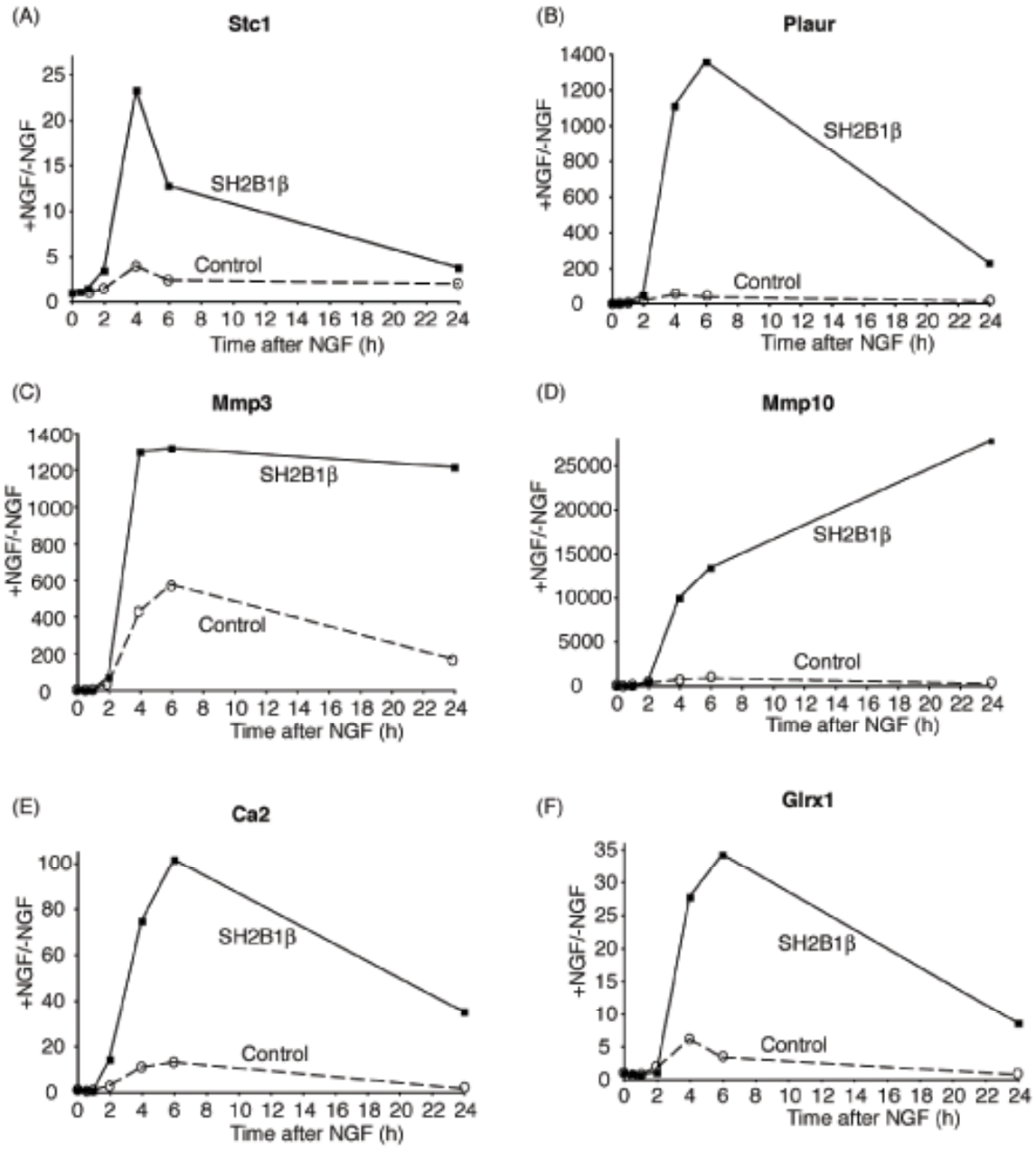


FIG. 3.4. Time course of SH2B1β enhancement of expression of representative NGF-regulated genes.

Control PC12 or PC12-SH2B1β cells were treated with 100 ng/ml NGF for 0, 30 min, 1h, 2h, 4h, 6h, or 24h. Relative gene expression levels were determined using QT-PCR, and normalized to levels of GAPDH gene expression.

Effect of SH2B1β on NGF-induced uPAR protein level and uPAR subcellular distribution. Strikingly, proteins encoded by three of the genes showing the greatest induction by NGF at 6 h as well as the greatest enhancement of that induction by SH2B1β or diminishment of that induction by SH2B1β(R555E) (uPAR, MMP3 and MMP10) fall in the same protease cascade (FIG. 3.5). In this cascade, uPAR binds the enzymatically inactive proform of urokinase plasminogen activator (pro-uPA), allowing it to be cleaved by cathepsins and thereby activated. The activated uPA proteinase subsequently cleaves inactive plasminogen to form enzymatically active plasmin which in turn cleaves inactive pro-MMPs to form enzymatically active MMPs. This cascade has been implicated in neurite outgrowth and more generally in cell differentiation, tissue remodeling, cell invasiveness and wound healing [reviewed in (191, 192)]. Because of the high level of both NGF and SH2B1β regulation of gene expression for uPAR, MMP3, and MMP10, the identification of Plaur as a primary response gene, and the previously documented critical role of uPAR in NGF-induced neuronal differentiation, we studied further the effect of SH2B1β on uPAR, MMP3 and MMP10. To confirm that the changes in NGF-induced Plaur gene expression levels seen in PC12-SH2B1β and PC12-SH2B1β(R555E) cells results in corresponding changes in the levels of uPAR protein, we examined levels of uPAR protein in control, PC12-SH2B1β and PC12-SH2B1β(R555E) cells by both western blot and immunocytochemistry. In western blots of lysates from control cells, 4h of NGF treatment transiently increased levels of uPAR protein (Fig 3.6A). In PC12-SH2B1β cells, NGF increased levels of uPAR protein at both 4 and 6 h, with the 6 h levels being significantly greater than in control cells. Compared to control cells, PC12-SH2B1β(R555E) cells showed reduced levels of both basal and NGF-induced uPAR protein at all time points. Levels of expression of SH2B1β and SH2B1β(R555E) were similar to each other and all 3 cell lines expressed similar levels of actin, indicating that the differences seen in uPAR levels were not due to differences in the amount of protein loaded. The

reason why the magnitude of the uPAR signal in PC12-SH2B1 cells relative to control PC12 cells was lower than what the gene expression studies would predict is not known. However, the fact that the difference in levels in NGF-induced protein expression between control and SH2B1β PC12 cells is greatest at 6 h raises the possibility that even greater differences would be detectable at later times.

To confirm the relative levels of NGF-induced uPAR expression and to determine whether SH2B1β affects the spatial distribution of uPAR, we immunostained control PC12, PC12-SH2B1β and PC12-SH2B1β(R555E) cells for uPAR after 0, 7h or 24h of 100ng/ml NGF. Immunostaining was performed under non-permeabilizing conditions to allow better visualization of uPAR associated with the plasma membrane. In all three cell lines, uPAR was observed at the cell surface in adhesion complex-like puncta (FIG. 3.6G, I, K, M, O), consistent with uPAR being a glycosyl-phosphatidyl inositol (GPI)-linked protein and its known interaction with proteins (e.g. integrins) in adhesion complexes [reviewed in (193)]. In all three cell lines, plasma membrane uPAR was present throughout the cell body of the neurite and at growth cones (FIG. 3.6 G, I, K, M, and a darker exposure of FIG. 3.6O, data not shown). NGF treatment increased levels of uPAR at the plasma membrane of PC12-SH2B1β cells at both 7 and 24 h (compare FIGS. 3.6G and 3.6I to 3.6E) as well as in control and SH2B1β(R555E) cells (data not shown). At 24 h following NGF addition, plasma membrane uPAR immunostaining was clearly greater in PC12-SH2B1β cells (FIG. 3.6L) and lower in PC12-SH2B1β(R555E) cells (FIG. 3.6O) than in control PC12 cells (FIG. 3.6K). The previously reported requirement of uPAR for NGF-induced neurite outgrowth (122) combined with the ability of SH2B1 to greatly enhance both expression of Plaur gene and plasma membrane levels of uPAR protein reported here suggest that SH2B1 enhances NGF-induced neurite outgrowth at least in part by mediating or enhancing the NGF-induced expression of Plaur.

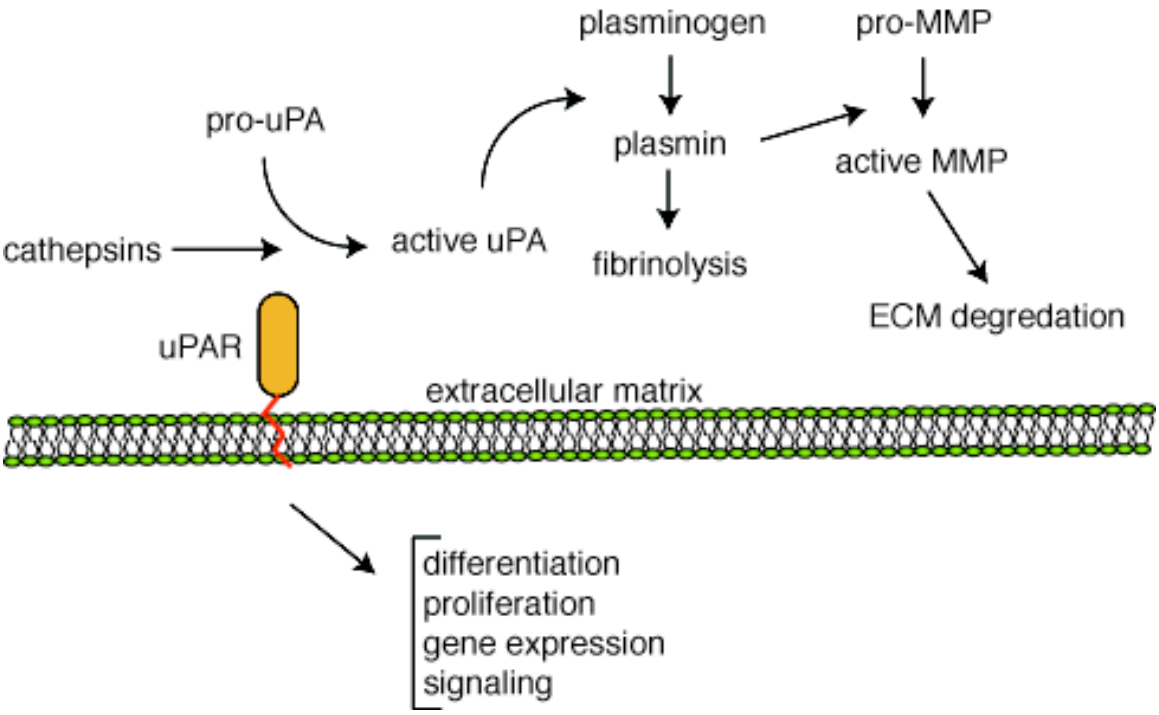


FIG. 3.5. Physiological functions of uPAR/MMP activation cascade.
Cell surface-localized uPAR, together with cathepsins, binds to pro-uPA and converts inactive pro-uPA to active uPA. The active uPA converts plasminogen to plasmin and the active plasmin cleaves and thereby activates MMPs. Active MMP has been shown to regulate extracellular matrix degradation.

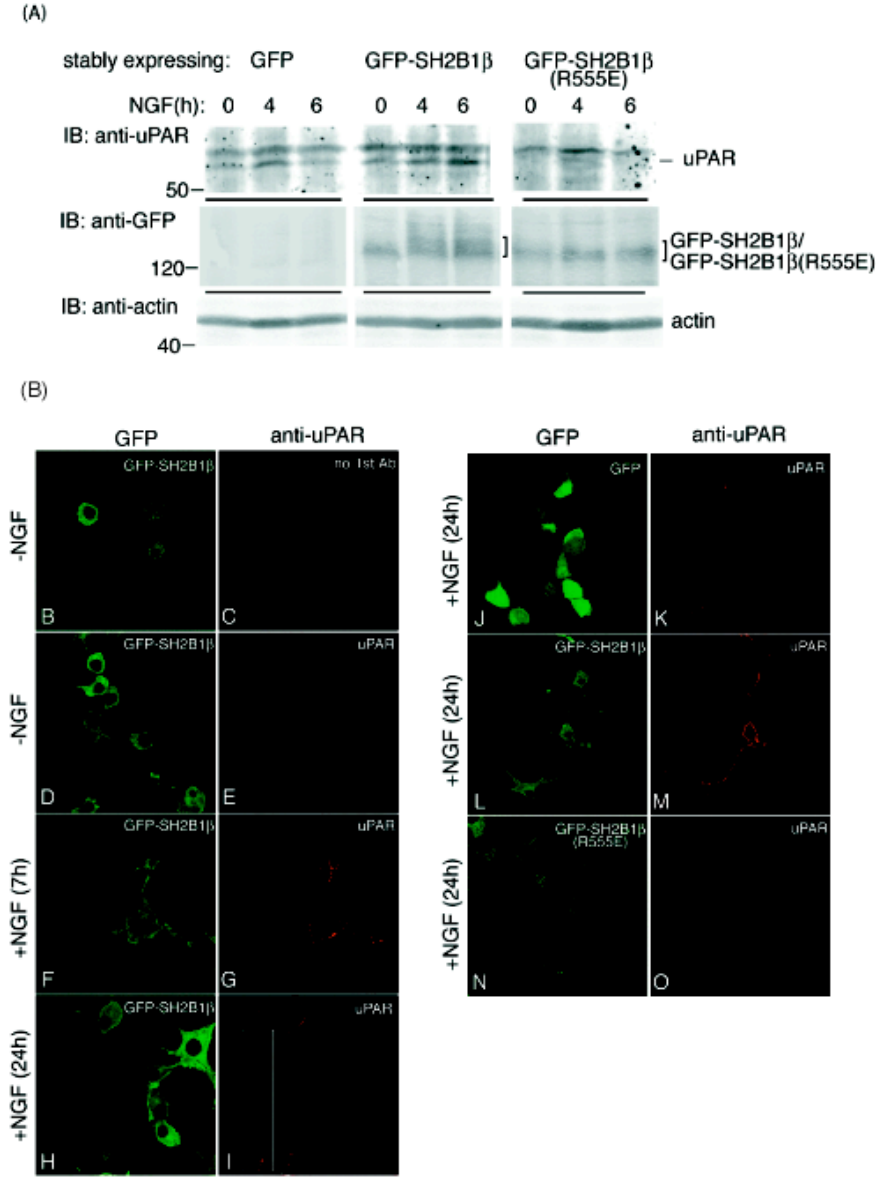


FIG. 3.6. SH2B1β enhances NGF-induced uPAR protein expression but does not affect the subcellular distribution of cell surface-localized uPAR. (A) PC12 cells stably expressing GFP, GFP-SH2B1β or GFP-SH2B1β(R555E) were incubated with 100 ng/ml NGF for 0, 4h, and 6h. Proteins in a whole cell lysate were separated by SDS-PAGE under non-reducing conditions and uPAR protein levels were determined by immunoblotting with antibody to rat uPAR. Expression levels of GFP-SH2B1β, GFP-SH2B1β(R555E) and actin were determined under reducing conditions by immunoblotting proteins in whole cell lysates with αGFP or αactin, respectively. The molecular weight markers are noted on the left (n=2). (B) PC12 cells stably expressing GFP, GFP-SH2B1β or GFP-SH2B1β(R555E) plated on Matrigel-coated coverslips were incubated with 100 ng/ml NGF for 0, 7h or 24h as indicated and fixed with 4% paraformaldehyde but not permeabilized before addition of monoclonal uPAR antibody and anti-mouse Alexa Fluor 555. Representative confocal images are shown. The insert in Panel I is an image of a different focal plane that focused more on the neurite. The experiment was repeated three times with similar results.

NGF-induced MMP 3/10 activity is elevated in PC12-SH2B1β cells and suppressed in PC12- SH2B1β(R555E) cells. We next examined the effect of NGF and SH2B1β on the protease activity of MMP3 and MMP10. Because of their similar size and substrate specificity and in the absence of immunoprecipitating antibodies that could distinguish between MMP3 and MMP10, we assayed MMP3 and MMP10 together. Conditioned medium was collected from cells treated with 100 ng/ml NGF for various times, concentrated, and assayed for the presence of MMP3 and MMP10 using a zymogram gel containing casein as substrate. When purified human MMP3 was used as a positive control, as expected, a cleared band was observed at Mr ~ 50,000 (non-reducing condition), consistent with MMP3 degrading the casein (FIG. 3.7A). Rat MMP3 migrates as a 60, 62 kDa doublet under reducing conditions. It was therefore expected to migrate slightly slower than human MMP3 (194). MMP3 and MMP10 are approximately the same size and cannot be differentiated in this assay. By 6 h of NGF treatment, control cells showed noticeable NGF-induced casein degrading activity at a molecular weight consistent for MMP3/10. This protease activity continued to increase with increasing incubation times (8h, 10 h) with NGF. Consistent with SH2B1β increasing NGF-induced levels of Mmp3/Mmp10 gene expression, this NGF-dependent protease activity was greater in magnitude in NGF-treated PC12-SH2B1β cells and substantially decreased in magnitude in NGF-treated PC12-SH2B1β(R555E) cells compared to control cells.

SH2B1β enhances NGF-induced neurite outgrowth of PC12 cells through Matrigel. Like uPAR activity, MMP3 activity has been associated with neurite outgrowth. However, unlike uPAR, its downregulation (by use of anti-sense RNA) did not affect the number or length of neurites produced by PC12 cells on a 2-dimensional tissue culture surface. Rather, downregulation of MMP3 blocked the ability of neurites to penetrate through a Matrigel barrier (123). Combined with the finding that SH2B1β enhances the activity of

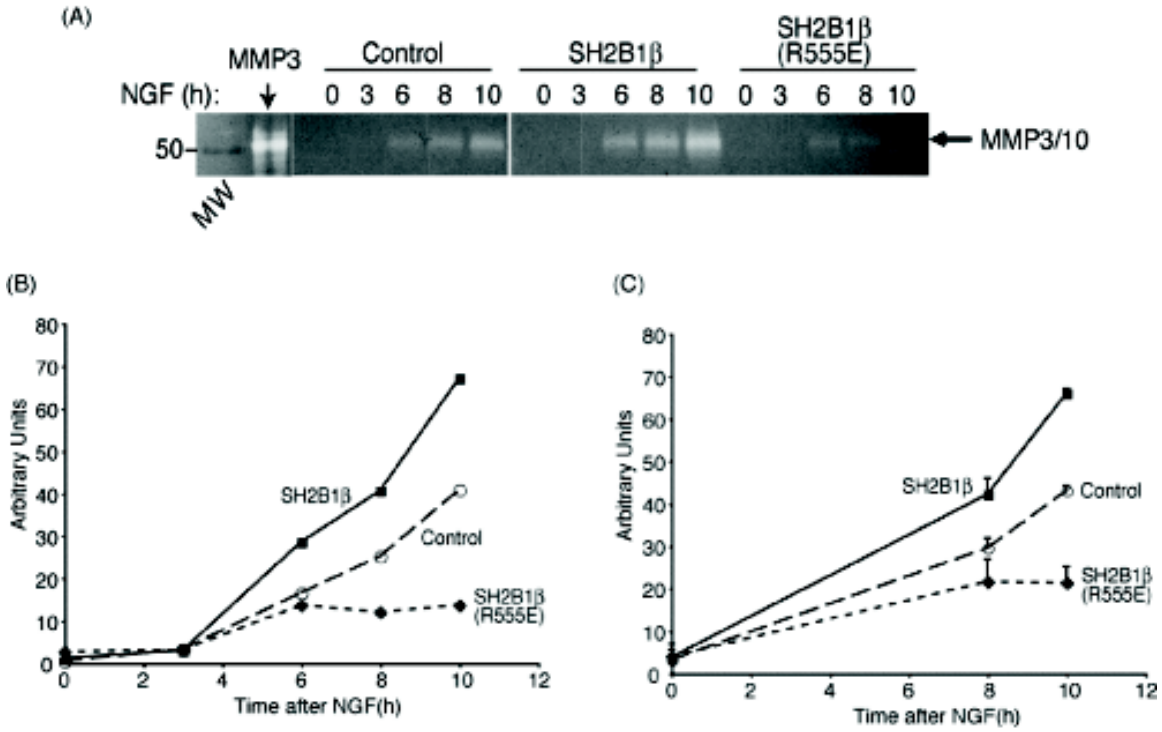


FIG. 3.7. SH2B1β enhances NGF-induced MMP3/MMP10 activity.

(A) Control PC12, PC12-SH2B1β or PC12-SH2B1β(R555E) cells were treated with 100 ng/ml NGF for 0, 3, 6, 8 and 10h. Proteins in concentrated conditioned medium were separated in casein-containing, pre-stained zymogram gels and then developed for 16h. A representative gel is shown. (B) The MMP3/10 activity in panel A was quantified by band intensity using NIH Image software. (C) NGF-induced MMP3/10 activity was quantified for 3 independent experiments. Means ± SEM are shown. MMP3/10 activity levels of the three cell lines are significantly different ($p<0.05$, one-tailed, paired Student T-test) at 10 h and by 8h, MMP3/10 activity in PC12-SH2B1β cells was elevated above control cells ($p>0.05$).

MMP3/10, this latter finding suggests that SH2B1β should enhance growth cone migration and invasion through an extracellular matrix barrier. To test this, control PC12 cells and PC12-SH2B1β cells were plated onto the *cis*-plane of a transwell membrane overlaid with growth factor-reduced Matrigel (FIG. 3.8A). NGF (100 ng/ml) was added every other day to the outer chamber for 4 days. Cells were then fixed and immunostained for neuronal β-tubulin. Neurites that traversed to the *trans*-plane of the membrane were counted. The number of neurites that invaded through the Matrigel to the *trans*-plane in cells expressing GFP-SH2B1β was found to be over twice (230%) the number of invading neurites from control cells (FIG. 3.8B). The neurons themselves were found not to traverse the trans-plane of the membrane.

Inhibition of SH2B1 suppresses NGF-induced expression of Plaur, Mmp3 and Mmp10. The ability of overexpression of wild-type SH2B1β to enhance and the dominant negative SH2B1β(R555E) to inhibit NGF-induced expression of Plaur, Mmp3 and Mmp10 indicates that endogenous SH2B1β regulates the NGF-induction of these three genes. To test this more directly, we used an RNA interference approach to stably reduce levels of endogenous SH2B1 in PC12 cells. As shown in FIG. 3.9A, the level of endogenous SH2B1 in cells stably expressing a 21-nucleotide long small hairpin RNA duplex targeted against SH2B1 (PC12-shSH2B1 cells) was reduced to < 50% the level in cells stably expressing the vector only (shControl cells), when normalized to levels of β-tubulin. As predicted from the experiments using PC12-SH2B1β and PC12-SH2B1β(R555E) cells, PC12-shSH2B1 cells exhibited substantially reduced NGF-induced neurite outgrowth compared to control cells (Maures, T, Chen, L and Carter-Su, C, manuscript in preparation). In support of endogenous SH2B1 enhancing NGF-induced expression of Plaur, Mmp3 and Mmp10, PC12-shSH2B1 cells exhibited substantially suppressed NGF-induced levels of expression of Plaur (by 56%), Mmp3 (by 47%) and Mmp10 (by 76%) compared to control cells (FIG. 3.9B), levels of reduction consistent with the level of

reduction in SH2B1β protein. Levels of GAPDH gene expression were not affected by NGF nor were they significantly different for the two cell lines, indicating that the differences in NGF-induction seen in Plaur, Mmp3 and Mmp10 expression in the different cell lines were not due to non-specific effects of the shRNAi on gene transcription or cell viability.

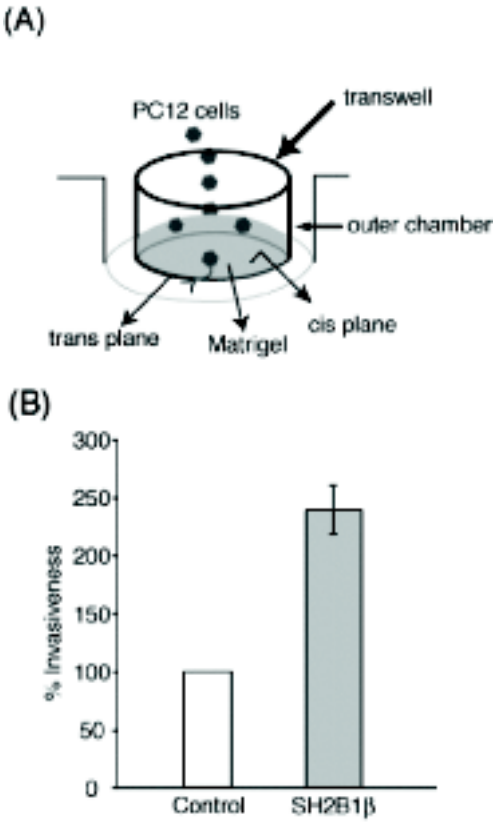


FIG. 3.8. SH2B1β enhances NGF-induced neurite invasiveness of PC12 cells.

(A) Schematic of transwell system used to measure neurite invasiveness. Cells were added onto growth factor-reduced Matrigel on the *cis*-plane of a transwell insert. Neurite invasiveness was assessed by counting the neurites appearing on the *trans*-plane of the transwell insert. (B) Control PC12 cells or PC12-SH2B1β cells were added to the Matrigel on the *cis*-plane of a transwell insert and 100 ng/ml NGF was added to the outer chamber. After 4 days, the cells on the insert were fixed and immunostained with anti-β tubulin and Alexa Fluor 555. Neurites that traversed to the *trans*-plane were counted. Means ± S.E.M. are shown for n=3.

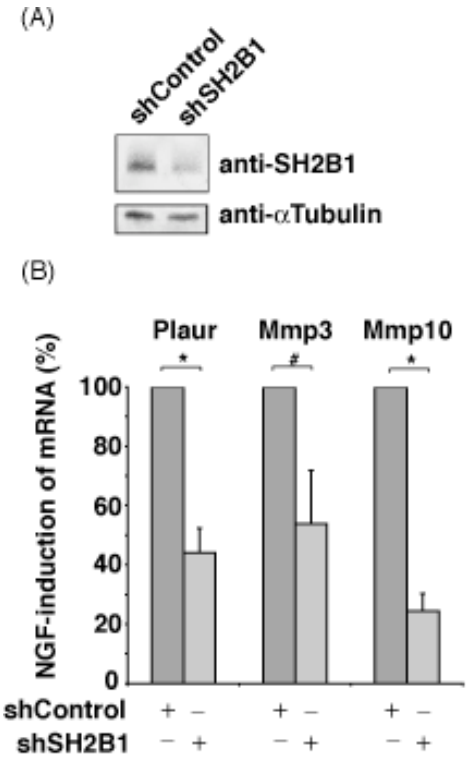


FIG. 3.9. SiRNA mediated knockdown of endogenous SH2B1β inhibits NGF-dependent induction of Plaur, Mmp3, and Mmp10.

(A) Proteins in lysates of PC12 cells stably expressing the control shRNA ("shControl") or shRNA targeted against SH2B1 ("shSH2B1") were separated by SDS-PAGE and immunoblotted with αSH2B1 and then anti-αtubulin as a loading control. (B) After incubation in serum-free medium overnight, PC12 cells expressing control shRNA or shRNA targeted against SH2B1 were incubated with or without 50ng/ml NGF for 6h. The NGF-dependent induction of mRNA for Plaur, Mmp3, and Mmp10 was quantified via QT-PCR. Gene expression in the presence of NGF was divided by expression in the absence of NGF, and then normalized to levels of GAPDH expression in the presence of NGF divided by expression in the absence of NGF. Results were then further normalized to those obtained using shControl cells. Means ± S.E.M. are shown for n = 3. Asterisks represent p values of <0.05 using a one-tailed, paired Student T-test. # denotes a p value of 0.06.

Discussion

Microarray analysis identifies novel NGF-responsive genes likely to be important for NGF-induced neuronal differentiation. In this study, we applied genome-scale gene expression analysis to identify SH2B1β-regulated genes and to gain insight into whether the adaptor protein SH2B1β enhances NGF-induced regulation of all NGF-sensitive genes, as predicted if SH2B1 simply potentiates NGF activation of TrkA, or whether it alters expression of a subset of NGF-sensitive genes as predicted if SH2B1 were acting downstream of TrkA. Because we were interested in the early events of NGF-induced neuronal differentiation, including neurite outgrowth, we looked at the gene expression profiles of the well-defined sympathetic neuron model PC12 cells before and after 6h treatment of NGF.

Although our main goal was to identify genes that are regulated by SH2B1β, our microarray analysis also identified over 1000 genes whose expression was substantially regulated by NGF, many more than identified previously. Several other studies have reported NGF-induced gene expression in large scale (195-202), however, most of these studies focused on the late response genes induced by 4 or more days of NGF treatment. The few studies looking at earlier time points identified a limited number of NGF-sensitive genes, the majority of which were also identified as NGF-sensitive genes in our microarray analysis. For example, Chou et al. (203) used gene expression profiling of PC12 cells at 1, 6 and 24 h after NGF treatment to identify nine genes out of 588 genes and ESTs that were highly regulated (> 300% or <33% of control) in response to NGF. Expression of the two genes (Mmp10 and Vgf encoding VGF nerve growth factor inducible/VGF8a protein/neurosecretory protein VGF precursor) found to be highly upregulated after 6 h treatment with NGF were also highly upregulated (7 and 38 fold respectively) by 6 h of NGF in our microarray study. Among the NGF-regulated genes identified by Brown et al.

(202) using targeted display to identify NGF-sensitive genes after 2, 4 and 6 h, our microarray data showed similar upregulation by NGF of Catna1 encoding α catenin, Odc1 encoding ornithine decarboxylase, Gsr encoding glutathione reductase, and Nup54 encoding p54 nucleoporin; and down-regulation of Gucy1b3 encoding β subunit of soluble guanylate cyclase and Chaf1A encoding p150 chromatic assembly factor. Finally, our microarray also identified as highly NGF-dependent six genes identified by Hershman's group (122, 201, 204) as being induced by 1-4 h of NGF but not EGF treatment using representational difference analysis: Arc (encoding activity-regulated cytoskeletal protein), Mmp13 (encoding MMP13/collagenase-3), Serpine1 (encoding plasminogen activator inhibitor-1 precursor/PAI-1), VH6/MKP-3/Dusp6 (encoding dual specificity phosphatase 6/Map kinase phosphatase 3), Plaur, and Nid67 (encoding NGF-induced differentiation clone 67 protein).

In addition to these previously identified NGF-induced genes, our microarray data identified close to 500 additional genes and ESTs whose expression was at least doubled with 6 h of NGF treatment and close to 150 additional genes and ESTs whose expression was at least tripled. More than 500 genes and ESTs showed an NGF-induced decrease in expression of 50% or greater, with over 100 showing a 66% or greater decrease. The NGF-sensitive genes identified in our study that were upregulated at least two-fold were overrepresented in the GO categories of morphogenesis, organogenesis, cell death, intracellular signaling and cell differentiation, consistent with NGF promoting differentiation and survival of sensory and sympathetic neurons. Those genes that were downregulated at least 50% by NGF were overrepresented in the categories of cell cycle, proliferation and migration, consistent with the known decrease in cell proliferation and movement that occurs during differentiation. Some of these NGF-regulated genes [e.g. genes encoding integrin α1; lectin, galactose binding, soluble 3 (198), bradykinin B2 receptor (199), Bip3 (205)] had been shown previously to be regulated by NGF in PC12 cells using a variety of techniques, although not always at such an early

time point (6 h) after NGF addition. Other genes (e.g. Glrx1 encoding glutaredoxin 1, Ca2 encoding carbonic anhydrase-II, and Stc1 encoding stanniolcalcin 1) were revealed for the first time to be highly responsive to NGF. Many of the proteins encoded by our newly identified NGF-regulated genes, including Glrx1, Stc1 and Ca2, had, however, been previously identified in neurons and in some cases, been implicated in neuronal differentiation and/or function. For instance, glutaredoxin 1 is thought to play an important role in maintaining nerve cell function in the presence of oxidative stress, at least in part by catalyzing the removal of glutathione from S-glutathionylated proteins [reviewed in (206)]. Since even small changes in the redox state of a cell have been shown to switch cells from proliferation to differentiation or vice versa [reviewed in (207)], our finding that NGF greatly upregulates glutaredoxin 1 expression raises the possibility that glutaredoxin 1 also plays an important role in NGF-induced neuronal differentiation. Proteins that regulate cellular pH, such as carbonic anhydrase II, are also thought to be especially important in the nervous system because electrical activity can elicit rapid changes in cellular pH (208). Our finding that carbonic anhydrase II, found in certain subsets of nerves, is rapidly and profoundly upregulated by NGF in differentiating PC12 cells and the finding of Dickens et al. (209) that tips of extending neurites appear to be more alkaline (by 0.2-0.3 pH units) than the cell body raise the possibility that carbonic anhydrase II plays a specific role in neurite outgrowth. Although not previously reported to be induced by NGF, stanniolcalcin-1, a glycoprotein hormone implicated in the regulation of calcium and phosphate homeostasis, has been reported to be induced during differentiation of a variety of cell types, including human neural crest-derived Paju cells (stimulated by phorbol esters) (210) and murine Neuro-2A neuroblastoma cells (stimulated by dibutyryl cAMP) (211). Decreasing its expression in Neuro-2A cells using anti-sense oligodeoxynucleotides reduces in neurite outgrowths the level of varicoses, a phenotypic marker of axon formation. This finding led to the suggestion that stanniolcalcin-1, which is found in neurons in human and adult mouse brain, is

involved in axonal formation. Clearly, knowledge and further exploration of these proteins and the proteins encoded by the large number of other genes newly found to be NGF-sensitive should greatly facilitate our understanding of how NGF induces neuronal differentiation and promotes neuronal survival.

SH2B1β enhances the NGF-regulation of a subset of genes likely to be involved in neuronal differentiation. Comparison of the gene expression profiles of control cells and cells stably expressing SH2B1β or the dominant negative SH2B1β(R555E) revealed a number of NGF-induced and -depressed genes that are differentially regulated by SH2B1β and SH2B1β(R555E). Some genes whose expression is substantially upregulated by SH2B1β have been reported previously to be expressed in neurons but were not known to be regulated by NGF (i.e. Stc1, Glrx1(a), Scn6a) (212-214). Other NGF-sensitive genes whose NGF stimulation is increased by SH2B1β (e.g. Arc, Fosl1/Fra1, Dusp6, Egr1, Plaur) have been shown previously to be regulated by NGF in neuronal cells (122, 201, 215). The gene products of a few (i.e. Plaur, Gap-43/Basp2) have even been implicated previously in NGF-induced neurite outgrowth (122, 216). For the six genes tested (Glrx1, Ca2, Stc1, Mmp3, Mmp10, Plaur), QT-PCR analysis confirmed both their upregulation by NGF and the ability of SH2B1β to increase the magnitude of that upregulation. QT-PCR analysis further revealed that for all 6 of the genes tested, SH2B1β significantly enhanced levels of expression at multiple time points after NGF addition, indicating that the SH2B1β-stimulated levels of expression seen at 6 h of NGF treatment in the microarray were not simply a consequence of a shift in the time course of NGF activation. Western blotting, activity assays, and/or immunocytochemistry indicated that increased expression of Plaur, Mmp3 and Mmp10 genes in PC12-SH2B1β cells results in increased protein expression of uPAR and MMP3/10 and that decreased gene expression of Plaur, Mmp3 and Mmp10 genes in PC12-SH2B1B(R555E) cells results in decreased protein expression of uPAR and MMP3/10.

It is important to note, however, that while the NGF-responsiveness of a number of NGF-sensitive genes was enhanced by overexpressing SH2B1, there were similar numbers of NGF-sensitive genes whose expression was not substantially altered by overexpressing SH2B1β. Genes in this latter category included some genes whose expression is considered critical for NGF-induced neuronal differentiation. For example, NGF-induced expression of Anxa2 encoding Annexin II/calpactin 1 heavy chain is thought to be critical for NGF-induced neuronal differentiation of PC-12 cells. Polyclonal and monoclonal antibodies directed against Annexin-II and overexpression of antisense Annexin-II mRNA inhibit NGF-induced PC12 neurite outgrowth on a plate and within a three-dimensional matrix (217). Annexin II is reported to be a co-receptor for plasminogen and tissue plasminogen activator (t-PA) that promotes and localizes plasmin generation near the cell surface (218, 219). Although in our microarray, NGF was observed to stimulate the expression of Anxa2 to three times the level seen in the absence of NGF, NGF-induction of expression was similar in control, PC12-SH2B1β and PC12-SH2B1β(R555E) cells. Like Plaur, Vgf, encoding VGF nerve growth factor inducible protein, is a primary response gene that is specific for NGF and not EGF and peaks in expression in PC12 cells after 2-8 h of NGF (122). Ret encoding the common receptor for GDNF family ligands is thought to be required for GDNF to instruct a subset of TrkA+ sensory neurons to adopt a nonpeptidergic sensory neuron fate (220). Although in control cells we observed NGF to substantially increase expression of both Vgf and Ret (by ~7 fold), the NGF-induced expression of these two genes was not altered in PC12-SH2B1β or PC12-SH2B1β(R555E) cells. Taken together, these findings suggest that SH2B1β enhances the expression of a critical subset of NGF-responsive genes implicated in neuronal differentiation.

SH2B1β enhances the ability of nerves to express uPAR and MMP3/10, which in turn enhances the ability of neurites to invade the extracellular matrix. When we looked at the top 5 genes whose NGF-induced expression at 6 h by microarray analysis was most highly upregulated in PC12-SH2B1β cells or

downregulated in PC12-SH2B1β(R555E) cells compared to control cells, we were impressed by the fact that three of the protein products (uPAR, MMP3 and MMP10) are involved in the same pathway of extracellular matrix degradation, a pathway critical for neurite outgrowth. What was even more striking is that the genes encoding these three proteins were also among the top 12 genes upregulated by NGF at 6 h in control cells. One of these genes (Plaur) is a known early response gene regulated by NGF (which promotes neuronal differentiation of PC12 cells) but not EGF (which does not promote neuronal differentiation of PC12 cells), shown previously to be essential for NGF-induced neurite outgrowth and biochemical differentiation (121, 122). These combined observations caused us to focus on uPAR, MMP3 and MMP10 as key mediators of the stimulatory effect of SH2B1 on NGF-induced neurite outgrowth.

The key features of NGF-dependent neuronal growth, such as the initiation and extension of the axon, are contingent upon the formation and motility of the growth cones. uPAR and MMPs 3 and 10 function together in a pathway to modulate the cell's degradation of the surrounding extracellular matrix (ECM), allowing the axon to "invade" or penetrate the extracellular matrix. uPAR is a GPI-linked protein that binds to and activates urokinase plasminogen activator (uPA) at the cell surface. uPAR has been implicated as being vital for NGF-induced differentiation of PC12 cells. Thus, in PC12 cells, anti-uPAR antibody to the extracellular domain of uPAR and anti-sense RNA targeted to uPAR message both block NGF-induced neurite outgrowth and appearance of biochemical markers of neuronal differentiation (121). uPAR is also thought to be an essential player for neuronal differentiation *in vivo*. uPAR is found in primary neurons and has been implicated in the differentiation of primary neurons. Hayden and Seeds (221) report that uPAR mRNA levels greatly increase during differentiation of cultured cells from mouse dorsal root ganglia (DRGs). The time course of the transient increase (maximum at 30 h) directly correlated with the differentiation of neurons and the formation of a neuritic network in the regenerating cultures. Levels of uPAR mRNA also increase in sensory neurons

after sciatic nerve crush in adult mice (222) and the neocortex of uPAR$^{-/-}$ adult mice has fewer GABAergic interneurons during embryonic and postnatal periods (223, 224).

Activated uPA converts plasminogen to plasmin, which subsequently cleaves inactive MMPs into active extracellular proteases. There are currently 28 identified enzymes in the MMP family, each displaying a propensity for the cleavage of specific substrates. MMP3 and 10 are secreted proteinases that are cleaved by plasmin; they are both capable of degrading a broad spectrum of substrates, including collagen I, III, IV, V and fibronectin (188). In response to signals like NGF, neurons position these extracellular matrix proteases to their growth cones. In PC12 cells, the time course of NGF-induced Mmp3 expression is largely coincident with neuronal differentiation (225) and MMP3 has been implicated in the motility of the growth cone (123, 226). Knocking down MMP3 in PC12 cells blocks NGF-induced neurite "invasion" or extension through Matrigel, suggesting that MMP3 is vital for axon outgrowth through the extracellular matrix. In support of a role of MMPs in axonal outgrowth *in vivo*, MMP3 and MMP10 are found in primary neurons. MMPs have also been implicated in penetration of DRG neurites through the extracellular matrix (227). Thus, in the most simplistic explanation of how uPAR, MMP3 and MMP10 mediate neurite outgrowth, NGF causes an increased transcription/translation of uPAR and MMPs, bestowing the cell with a greater proteolytic potential which allows the growth cones to cut through the ECM, clearing a pathway for the neurite. The ability of SH2B1β to greatly increase the expression of uPAR and increase and prolong the expression of both MMP3 and MMP10 suggests that SH2B1β is required for the degradation of the ECM required for axonal outgrowth of NGF sensitive nerves. This is supported by our finding that both overexpression of the dominant negative SH2B1β(R555E) and reduction of expression of endogenous SH2B1 using shRNA to SH2B1 substantially decrease the expression of uPAR, MMP3 and MMP10. It is further supported by the finding that cells overexpressing SH2B1β both increased the amount of enzymatically active MMP3/10 over levels

associated with control PC12 cells in response to NGF as well as NGF-induced neurite “invasion” through Matrigel.

However, the contribution of uPAR and MMPs, and thus, SH2B1, to neurite outgrowth may extend beyond this simple explanation. The requirement for functional uPAR during NGF-dependent differentiation of PC12 cells is transient and essential only for the initial hours of NGF exposure (121, 122). Furthermore, not only does depletion or inhibition of plasma membrane uPAR block NGF-induced neurite outgrowth in a culture plate, but it also blocks biochemical differentiation assessed as expression of COX-1 and type II sodium channel and expression of both MMP3 (Transin) and MMP1 (Collegenase I) (122). These observations suggest that uPAR is an immediate early gene product of NGF whose function may be required for induction of a wave of secondary response genes important for differentiation. Indeed, uPAR has also been reported in other cell types to activate a variety of intracellular signaling pathways and increase levels of a variety of signaling molecules. The pathways and molecules include JAK1/Stat1 (228), MEK/ERK (229), the Src family kinase hck (230) and protein kinase Cε (231) pathways as well as diacylglycerol (232, 233), cAMP (234), calcium released from internal stores (235) and inositol phosphate turnover (236). In other cell model systems, uPAR has been reported to form stable complexes with integrins that alter the adhesive properties of the cells (237, 238). Thus, NGF-induced neuronal differentiation and enhancement of that differentiation by SH2B1β may also require uPAR activation of cellular signaling pathways.

In a similar vein, both axons and growth cones appear to require MMPs to not only extend into the extracellular matrix, but also to remove inhibitor proteins and process guidance cues [reviewed in (239)]. MMP3 has been implicated in synaptogenesis (240) and found to confer neuronal survival properties by removing FasL from the neuronal surface, thereby protecting against Fas-induced apoptosis (241). Thus, in intact neurons, as a consequence of promoting and prolonging the NGF-induced expression of Mmp3 and Mmp10,

SH2B1β may not only be enhancing neurite invasion of the extracellular matrix, it may also be enhancing neuronal survival and affecting synaptogenesis. A specific role for SH2B1 in neuronal survival is consistent with the finding of Qian et al. (34) that antibodies to SH2B1α introduced into dissociated sympathetic neurons maintained in the presence of NGF promote cell death and that introduction of a dominant negative form of SH2B1α into explants of sympathetic ganglia maintained in the presence of NGF result in axonal degeneration.

Cellular mechanism by which SH2B1β enhances expression of uPAR and MMP3. Mmp3 expression has been shown to be dependent upon uPAR expression (122, 201), making it likely that SH2Bβ1 enhancement of MMP3 expression is secondary to SH2B1β enhancement of uPAR expression. The molecular mechanism by which SH2B1β enhances NGF-induced Plaur gene expression is unknown. As discussed previously, one hypothesis is that SH2B1 increases NGF-induction of TrkA (96), which would be expected to result in an increase in all cellular responses to NGF, including expression of Plaur. However, our data showing that SH2B1β increases expression of only a subset of NGF-induced genes and previous data showing that NGF-induction of TrkA is not enhanced in PC12-SH2B1β(R555E) cells (33, 79) argue against this being the primary explanation for how SH2B1β stimulates expression of Plaur. Recent findings suggest that NGF causes the recruitment of c-Fos and JunB to an AP-1 regulatory element within the uPAR promoter (242), raising the possibility that SH2B1 somehow facilitates that process either directly in its role as an adapter protein or indirectly by promoting expression of c-Fos or JunB.

The work of Qian and colleagues (34)((96) is consistent with SH2B1 enhancing NGF activation of ERKs 1 and 2, which would be expected to increase the expression of c-Fos. Arguing against this hypothesis, however, is that while overexpression of SH2B1β has a modest enhancing effect on NGF activation of phosphorylation of ERKs 1 and 2 (33, 79), neither overexpression of SH2B1β(R555E) (33) nor reduction of endogenous SH2B1 (Maures, T, Chen, L

and Carter-Su, C, manuscript in preparation) inhibits NGF-induced ERK phosphorylation. In support of the conclusion that SH2B1β does not enhance NGF-induced Plaur expression and neuronal differentation solely by enhancing NGF-induced activation of TrkA or ERKs 1/2, glial-derived neurotrophic factor (GDNF)-induced neuronal differentiation of PC12 cells stably overexpressing GDNF receptor α1 and RET is also enhanced by overexpression of SH2B1β but suppressed by overexpression of SH2B1β(R555E) and by reduction of endogenous SH2B1 using shRNA to SH2B1(35). However, GDNF stimulation of phosphorylation of Erks 1 and 2 and Akt was not appreciably suppressed by overexpression of SH2B1β(R555E).

Taken together, these findings suggest that SH2B1 enhances both NGF and GDNF-induced neuronal differentiation at a point downstream of or parallel to ERKs. The additional findings that SH2B1β enhancement of NGF-induced Plaur expression and neurite outgrowth appear to require nuclear cytoplasmic shuttling of SH2Bβ1 [(79); Maures, T, Chen, L and Carter-Su, C, manuscript in preparation] open up the possibility of a novel pathway that requires SH2B1 to cycle through the nucleus.

Conclusion. In conclusion, our microarray analysis of PC12 cells treated with or without NGF for 6 h identified ~ 1000 genes and ESTS whose expression was increased or decreased by NGF by a factor of 2 or more, many of which were not previously recognized as being NGF responsive genes. NGF-induced regulation of a substantial subset of these genes was enhanced by overexpressing wild-type SH2B1β and/or suppressed by overexpressing dominant negative SH2B1β(R555E). Another subset of NGF-sensitive genes implicated previously in NGF-induction of neuronal differentiation were unaffected by overexpression of SH2B1β or SH2B1β(R555E), suggesting that SH2B1β acts primarily downstream of TrkA. Three of the upregulated genes (Plaur, Mmp3 and Mmp10) were noted to be particularly sensitive to both NGF and SH2B1β. Their regulation by both NGF and SH2B1β at the gene level was confirmed by QT-PCR

and at the protein level by immunoblotting, immunocytochemistry, and/or functional assays. The protein products of these three genes lie in the same functional pathway of extracellular matrix degradation critical for neurite outgrowth; uPAR has been shown previously to be critical for NGF-induced neuronal differentiation and MMP3 for NGF-induced neurite growth through a 3-dimensional matrix. These observations suggest that uPAR, MMP3 and MMP10 are likely to play a critical role in the ability of SH2B1 to facilitate NGF-induced neuronal differentiation and neurite outgrowth. A critical role is further suggested by our findings that depletion of endogenous SH2B1β suppresses the ability of NGF to enhance expression of all three genes and that overexpression of SH2B1β promotes neurite extension through a three dimensional matrix. The additional finding that nuclear cytoplasmic shuttling of SH2B1β is required for SH2B1β to promote NGF-dependent neuronal differentiation makes it intriguing to speculate that nuclear SH2B1β directly facilitates expression of a subset (e.g. Plaur) of NGF-sensitive genes.

This Chapter has been published in **Molecular Endocrinology** (2008) 22(2) pgs. 454-476, under the title "SH2B1β (SH2-Bβ) Enhances Expression of a Subset of Nerve Growth Factor-Regulated Genes Important for Neuronal Differentiation Including Genes Encoding UPAR and MMP3/10" by Linyi Chen, Travis J. Maures, Hui Jin, Jeffrey S. Hou, Shafaat A. Rabbani, Jessica Schwartz, and Christin Carter-Su. Linyi Chen provided FIGs 3.2, 3.3A and B, 3.5, 3.6-3.8, and Table 3.5. I provided FIGs 3.1, 3.3C, and 3.9. Linyi Chen and I collaborated on FIG 3.5 and Tables 3.1-3.5. Linyi Chen, Travis J. Maures contributed equally to this work.

Chapter 4

Nucleocytoplasmic Shuttling of the Adapter Protein SH2B1β (SH2-Bβ) is Required for Nerve Growth Factor (NGF)-dependent Neurite Outgrowth and Enhancement of Expression of a Subset of NGF-responsive Genes

Abstract

The adapter protein SH2B1 (SH2-B, PSM) binds to active nerve growth factor (NGF) receptor TrkA and enhances NGF-mediated differentiation of preneuronal cells. Our previous finding that SH2B1β contains a nuclear export sequence (NES) and cycles between the cytoplasm and nucleus raised the possibility that nuclear localization of SH2B1β facilitates NGF-dependent neurite outgrowth. Here we used deletion and substitution mutants to map a functional monopartite nuclear localization signal within SH2B1. Expression of the nuclear localization defective SH2B1β(mNLS) inhibited NGF-dependent neurite outgrowth without inhibiting NGF-induced activation of TrkA and ERKs 1 and 2. Disruption of SH2B1β translocation into or out of the nucleus abolished the ability of SH2B1β to enhance transcription of the NGF-responsive genes encoding urokinase plasminogen activator receptor (uPAR/Plaur), matrix metalloproteinase 3 (Mmp3), and matrix metalloproteinase 10 (Mmp10). Nuclear translocation of the highly homologous family member SH2B2 (APS) was not observed, indicating that the ability to translocate to the nucleus is specific to SH2B1. Depleting endogenous SH2B1 using short hairpin RNA against SH2B1 proportionately blocked NGF-dependent neurite outgrowth, but did not impede NGF-mediated phosphorylation of Akt or ERKs 1 and 2. Together, these findings

suggest that SH2B1β promotes NGF-mediated neuronal differentiation at least in part by directly facilitating enhancement of NGF-mediated gene expression.

Introduction

Ligand activation of the TrkA receptor tyrosine kinase by nerve growth factor (NGF) initiates several well studied signaling pathways, which ultimately mediate survival as well as the development and target innervation of both sympathetic and sensory neurons. The range of physiological responses governed by the activation of TrkA underscores the importance of determining the molecular mechanisms responsible for these critical physiological outcomes. Much of our current understanding about NGF-induced (dependent) neuronal differentiation and the responsible signaling pathways comes from work using the PC12 cell model. Derived from a rat adrenal pheochromocytoma, PC12 cells cease proliferation in response to NGF, exhibit somatic hypertrophy, and acquire neurites (16). The resulting NGF-dependent morphological and biochemical changes of the PC12 cell are highly analogous to that of a true sympathetic neuron as the NGF-treated PC12 cells express neuronal specific genes (17) and are capable of forming synapses with primary neurons from rat cortex (18). Additionally, en route to NGF-mediated differentiation, the PC12 cells develop a dependence on NGF for survival as has been documented for both sympathetic and sensory neurons (180).

Initiation of NGF-dependent neuronal differentiation begins with the binding of NGF to TrkA, which activates TrkA, resulting in autophosphorylation of TrkA at several tyrosines. These newly and transiently phosphorylated residues function to recruit a number of different signaling molecules and initiate a variety of signaling pathways, including pathways involving phospholipase C-γ (PLC-γ); NFκB; phosphatidylinositol-3-kinase (PI3K)/Akt; Ras/Raf/MEK/extracellular regulated kinases (ERKs) 1 and 2; Jun N-terminal kinase (JNK); p38; and atypical protein kinase C (reviewed in (19, 20)). The cell's ability to specifically induce and propagate the appropriate signaling pathways is thought to arise, at least in part, from interactions of activated TrkA with specific adaptor and/or

scaffolding proteins. Indeed, the NGF-dependent binding between TrkA and the scaffolding protein FRS2 has been shown to form a complex of proteins in PC12 cells, including Crk, C3G, Rap1 and B-Raf (31, 243) that leads to the prolonged phosphorylation of ERKs required for differentiation (27). Epidermal growth factor (EGF) activation of EGF receptor, on the other hand, is unable to recruit FRS2 and results in a transient activation of ERKs, leading to the proliferation of PC12 cells instead of their differentiation (31).

We and others identified the putative adapter protein SH2B1 (SH2-B, PSM) as one of the signaling proteins that bind to the NGF-activated form of TrkA (33, 34). NGF promotes the rapid association of SH2B1 with TrkA and subsequent phosphorylation of SH2B1 on tyrosines as well as serines/threonines (33, 68). SH2B1 belongs to a family of adapter proteins that includes APS (SH2B2) and Lnk (SH2B3) (44-46, 244). Each of the family members contains several proline-rich domains, at least one N-terminal dimerization domain (DD), a pleckstrin homology (PH) domain, and a C-terminal Src homology (SH2) domain (183) through which SH2B1 and SH2B2 are thought to interact with activated TrkA(34) (33). Splice variation leads to the formation of four SH2B1 isoforms, α, β, γ, and δ, which differ only in their C-termini after the SH2 domain (37) (46). The overexpression of both SH2B1β and SH2B1γ enhances NGF-dependent neurite outgrowth in PC12 cells (34) (33). However, the molecular mechanism by which SH2B1 enhances neurite outgrowth remains largely unknown. Previous efforts to elucidate this mechanism have been largely centered on the interaction between SH2B1 with TrkA. The apparent membrane localization of SH2B1β (68) and its recruitment to NGF-activated TrkA (33) support a mechanism in which SH2B1 enhances the kinase activity of TrkA and/or recruits essential NGF effector molecules to the activated receptor complex (34, 96), thereby driving the prolonged activation of ERKs required for NGF-dependent differentiation (27). However, expression of SH2B1β containing a point mutation within its SH2 domain critical for phosphotyrosine-dependant binding to TrkA (SH2B1β(R555E)), blocks NGF-dependent differentiation without affecting NGF-

induced phosphorylation of TrkA, PLC-γ, Akt, ERK1 or ERK2 (33, 245). This finding indicated that the dominant negative effect of overexpressed SH2B1β(R555E) on NGF-dependent neurite outgrowth is unlikely to be a result of reduced TrkA activation or a diminished ability of TrkA to phosphorylate the substrates responsible for the Shc/Ras/MEK/ERK cascade, PI3K/Akt, or PLCγ pathways. These results suggested that SH2B1β might facilitate NGF-dependent differentiation through a novel pathway at a point downstream of or parallel to ERKs 1 and 2.

Indeed, another layer of complexity was evidenced by the finding that SH2B1β contains a functional nuclear export sequence (NES) (79). Overexpressed SH2B1β as well as endogenous SH2B1 was found to accumulate in the nucleus of cells incubated in the presence of a nuclear export inhibitor, leptomycin B (LMB) (79), indicating that SH2B1β undergoes nucleocytoplasmic shuttling. This raised the possibility that nuclear localization of SH2B1 is required for its stimulatory effects on NGF-induced neuronal differentiation.

Recently, using microarray analysis of PC12 cells, we established that the overexpression of SH2B1β leads to altered transcription of only a specific subset of NGF-responsive genes, including uPAR (Plaur), Mmp3 (stromelysin-1; transin-1), and Mmp10 whose gene products encode urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 3 (MMP3), and matrix metalloproteinase 10 (MMP10) (124). Interestingly, uPAR present in the plasma membrane of both the cell body and neurites (79) has been shown to be required for NGF-mediated neuronal differentiation (121, 122) and MMP3, present in growth cones of NGF-treated PC12 cells, has been implicated in neurite penetration through the extracellular matrix (123). Additionally, uPAR, MMP 3, and MMP 10 are all situated in the same proteolytic cascade responsible for extracellular matrix degradation required for neurite penetration through the extracellular matrix) (reviewed in (124)). Overexpression of the dominant negative SH2B1β(R555E) blocks both NGF-dependent neurite outgrowth as well

as NGF-induced transcription of the genes encoding uPAR, MMP3, and MMP10 (124). These results suggest that SH2B1β mediates NGF-induced neuronal differentiation and neurite outgrowth at least in part by enhancing NGF-dependent expression of uPAR, MMP3, and MMP10. In light of these findings, we questioned whether the nucleocytoplasmic shuttling by SH2B1β is required for SH2B1β enhancement of NGF-induced neuronal differentiation and the ability of SH2B1β to facilitate transcription of the genes encoding uPAR, MMP3, and MMP10.

Herein, we provide the first characterization of a nuclear role for SH2B1β. Specifically, we demonstrate that endogenous SH2B1β is required for NGF-dependent neurite outgrowth, although nonessential for NGF activation of Akt and ERKs 1 and 2. Deletion and mutational analysis revealed that SH2B1β contains a monopartite nuclear localization sequence (NLS) in addition to its previously reported NES. The adjacent motifs of the NLS and the NES direct the cycling of SH2B1β between the nucleus and cytoplasm. Expression of a nuclear import defective SH2B1β inhibits NGF-dependent neurite outgrowth to the same extent as SH2B1(R555E). Interestingly, mutation of the SH2 domain of SH2B1β renders it unable to translocate to the nucleus. Using QT-PCR, we show that NGF-dependent transcription of uPAR, Mmp3, and Mmp10 is enhanced in cells overexpressing SH2B1β(WT), but not in cells expressing SH2B1β(R555E), or the nuclear import mutant of SH2B1β, and substantially decreased in cells expressing the nuclear export mutant of SH2B1β. Collectively, these data indicate that SH2B1β cycling between the nucleus and cytoplasm is required for both the enhanced NGF-induced transcription of uPAR, Mmp3, and Mmp10 and for NGF-dependent neurite outgrowth. In turn, this finding suggests that SH2B1β promotes NGF-mediated neuronal differentiation at least in part by directly facilitating enhancement of NGF-mediated gene expression.

Materials and Methods

Antibodies and reagents. Polyclonal antibody to rat SH2B1 (αSH2B1), kind gift of Dr. Liangyou Rui (University of Michigan), was raised against an SH2B1β glutathione S-transferase fusion protein and used at a dilution of 1:1000 for Western blotting (93). Antibodies that recognize the following proteins were used for Western blotting at a dilution of 1:1000: phospho-44/42 MAPK that recognizes both ERK1 and ERK2 that are doubly phosphorylated on T202/Y204 (apERK; E10), total Erk (aERK), and phospho-Akt (Ser473) (αpAkt-Ser473) from Cell Signaling Technology (Beverly, MA), and phospho-TrkA (Tyr 490) (αpTrkA(Tyr490)) (catalog item T9691) from Sigma-Aldrich (St. Louis, MO). IRDye800 conjugated, affinity purified anti-GFP (Rockland Immunochemicals Inc., Gilbertsville, PA) was used at a dilution of 1:20,000 to visualize GFP. IRDye 800 and IRDye 700 conjugated affinity purified anti-mouse IgG and anti-rabbit IgG (Rockland) and Alexa Fluor 680-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) were used at a dilution of 1:20,000. Ascites containing anti-mouse myc monoclonal antibody, produced by the Michigan Diabetes Research and Training Center Hybridoma Core, was used for immunostaining (dilution of 1:1000). Anti-myc staining was visualized using Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen). NGF and rat-tail collagen I were purchased from BD Bioscience (San Diego, CA). Fluorescein was from Bio-Rad (Hercules, CA) and SYBER Green I was from Sigma-Aldrich Chemical Co. (St. Louis, MO). TaqMan RT-PCR kit (Cat#N808-0234) was purchased from Applied Biosystem (Roche, Indianapolis, IN).

Plasmids. All SH2B1β cDNAs were subcloned into pEGFP C1 (CLONTECH Laboratories, Inc.). cDNAs encoding GFP-tagged SH2B1β, SH2B1β(198-268), and SH2B1β(R555E) have been described previously (33,

79). GFP-SH2B1β(147-198) was created from the GFP-SH2B1β(WT) parental plasmid using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Two BamH1 restriction sites (at 147 and 198) were put into SH2B1β(WT) cDNA, flanking the desired region to be deleted. The construct was digested with BamH1 and the resulting insert was omitted during re-ligation. The primers (sense strand, mutations in lower case) used for generating the deletion in GFP-SH2B1β(147-198) was (5'-CAACGTCCTCAAAGCCGggatcCAAGAAACGCTTCTCCC-3') for site 147 and (5'-GTTCTAGGTGGAAACggatcCTCCAACTCCTCTGGTGGT for site 198. GFP-SH2B1β(147-268) was created in a manner similar to the previously mentioned GFP-SH2B1β(147-198). The primers (sense strand, mutations in lower case) used to generate the BamH1 mutation at site 268 was (5'-CCTGACCCAGCAGGAtccGGTCGTGGAGGAGGG-3'). Critical basic residues K148, K150, K151, and R152 were mutated to alanines in the mutant cDNA encoding GFP-SH2B1β(mNLS), which was created from the GFP-SH2B1β(WT) expression vector using site directed mutagenesis. The primer (sense strand, mutations in lower case) used for GFP-SH2B1β(mNLS) was (5'-CGTCCTCAAAGCCGgcGCTCgcGgcAgcCTTCTCCCTCCGC-3'). cDNA encoding GFP-SH2B1β(mNLS+ΔNES) was similarly created through site-directed mutagenesis using GFP-SH2-Bβ(198-268) and the primers above. The cDNA encoding myc-tagged rat SH2B2 was kindly provided by Dr. David D. Ginty (Johns Hopkins University). To create myc-SH2B2(ΔNES), two HindIII sites were introduced into myc-SH2B2(WT) at amino acid positions 167 and 184 using site-directed mutagenesis. Primers used to introduce HindIII sites were (5'-GAGCCTCGTGACAAgcttACGCGACGTCTG-3') and (5'-GCAGCCAAAGTGaAGcTtGTGGACATCCAGCGC-3'), respectively. The resulting myc-SH2B2 mutant contained two HindIII sites flanking the putative NES. The construct was digested with HindIII restriction enzyme (New England Biolabs, Boston, MA), purified, and then re-ligated. The four residues within the

putative NLS of myc-SH2B2 were mutated to alanines, using site-directed mutagenesis from Stratagene. The primer used to produce myc-SH2B2(mNLS) was (5'-CACGTGGCTACCgcGGCCgcTGTCgcCgcAGGCTTCTCACTG-3'). All mutations were verified by DNA sequencing.

Stable cell lines and cell culture. The parental PC12 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). Pools of PC12 cells stably expressing GFP-SH2B1β(mNLS) and GFP-SH2B1β(R555E) were made as described previously (79). The PC12 cell lines stably expressing GFP, GFP-SH2B1β(WT), GFP-SH2B1β(R555E), and GFP-SH2B1β(NLS) were cultured as described previously (79). PC12 cells were plated on collagen-coated plates (0.1 mg/ml rat tail collagen in 0.02 N acetic acid) and grown at 37°C in 10% CO2 in normal growth medium consisting of; Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen), containing 10% heat-inactivated horse serum (ICN), 5% fetal bovine serum (Invitrogen), 1 mM L-glutamine, and 1 mM antibiotic-antimycotic (Invitrogen).

Silencing of SH2B1 gene. SH2B1 siRNA vector was constructed by inserting an oligonucleotide containing the SH2B1 sequence (5'-CATCTGTGGTTCCAGTCCA-3') corresponding to nucleotides 1771-1789 of rat SH2B1 (GenBankTM accession number AF047577) into the pSuper retro vector containing the puromycin resistance gene (pSuper retro puro) (OligoEngine). A pSuper vector containing a non-targeting siRNA with a low sequence similarity to known genes was used as a control. The SH2B1 and control siRNA vectors were transfected into subconfluent PC12 cells using a BioRad Gene Pulser Xcell electroporator (400V, 500μF, 0.4 cm cuvette). After 14h, cells were washed with 1X phosphate-buffered saline (PBS) pH 7.4 and fresh growth medium was added. Twenty-four hours later, growth medium containing 5 μg/ml puromycin was added to the cells and selected for pSuper positive PC12 cells for 30 days.

Differentiation of PC12 cells. PC12 cells were plated on six-well, collagen-coated plates. The cells were grown in differentiation medium (DMEM containing 2% horse serum, 1% fetal bovine serum) and 50 ng or 100 ng of NGF per ml was added. Cells were replaced every 2 days until cellular differentiation was assessed. Cells with neurite length at least twice the diameter of the cell body were scored as differentiated cells. The percentage of differentiated cells was determined by dividing the number of morphologically differentiated cells by the total number of cells counted.

Immunolocalization. PC12 cells were transfected using a Gene Pulser Xcell electroporator (400V, 500μF) (Bio-Rad) in a 0.4 cm cuvette. After 5 h, cells were washed with PBS and fresh growth medium was added. Cells were incubated for 24 h before cells were fixed with 4% paraformaldehyde. Coverslips were mounted onto slides with Prolong (Molecular Probes). Cells were incubated with 2 ng of DAPI per ml for 10 min to visualize nuclei. The subcellular distribution of the various GFP-SH2B1β proteins was determined by GFP fluorescence. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200) with either 20X or 40X objectives. Images were captured using a CoolSnap HQ digital camera (Roper Scientific) and viewed using MetaVue imaging software (Molecular Devices, Sunnyvale, Ca).

RNA preparation and QT-PCR. Total RNA was isolated from control and NGF-treated PC12 cells using Stat60 (Tel-Test, Inc.) according to the manufacturer's instructions. RNA from four experiments was prepared and the quality of each RNA was checked by optical density. cDNA was generated from each RNA preparation using One-Step RT-PCR Kit with SYBR Green (Bio-Rad, catalog item 170-8892) according to the manufacturer's instructions. NGF-induced gene expression of uPAR, Mmp3, Mmp10 was assessed by QT-PCR using SYBR green I and the iCycler system with iCycler iQ Real Time Detection System software (Bio-Rad). Primer sequences were designed using

PrimerExpress software, as described in (124). Amplicons generated from each primer pair were 50-52bp. The PCR well volume of each sample was normalized with fluorescein. All readings were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are shown as a ratio of gene expression in NGF-treated cells to gene expression in control (no NGF) cells.

Cell lysis, immunoprecipitation and immunoblotting. Cells were washed three times with chilled PBSV (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na3VO4, pH 7.4) and solubilized in lysis buffer (50 mM Tris, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na3VO4, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 25 mM NaF. The solubilized material was centrifuged at 16,750 x g at 4oC for 10 min. The lysates were boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl, 10% SDS, 10% b-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, pH 6.8). Equal amounts of the solubilized proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, immunoblotted with the indicated antibody, and detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Immunoblots were quantified using LiCor Odyssey 2.0 software and normalized to the levels of α-tubulin (FIG. 4.1) or total ERK (FIG. 4.7).

Results

SH2B1 is required for NGF-dependent differentiation of PC12 cells.

In our ongoing attempts to elucidate the mechanism by which SH2B1 enhances NGF-dependent neurite outgrowth, we wanted to determine whether endogenous SH2B1 is required for NGF-dependent neurite outgrowth. To do so, we used an RNA interference approach to stably reduce endogenous levels of SH2B1 in PC12 cells. As demonstrated previously (124) and in FIG. 4.2, endogenous SH2B1 levels in cells stably expressing a 21-nucleotide long small hairpin RNA targeted against all isoforms of SH2B1 (shSH2B1) was reduced to less than 50% the level in cells stably expressing the vector only (shControl), when normalized to the levels of α-tubulin (based on quantification of 3 experiments). We compared the ability of the shControl and shSH2B1 PC12 cell lines to differentiate in the presence of NGF. The shControl and shSH2B1 cell lines were incubated in serum-free medium overnight before treatment with 50 ng/ml NGF for four days. Cells were assessed for differentiation on each of the four days. They were considered differentiated if they had neurites at least twice the length of their cell diameter. As shown in FIG. 4.1, there was little difference in neurite outgrowth between the two cell lines on day 1. However, every day thereafter (days 2, 3, and 4), we observed approximately half as many neurites in the SH2B1 knockdown cells as compared to the control cells, a decrease similar to that of the SH2B1 protein levels. These results indicate that endogenous SH2B1 is required for NGF-induced neurite outgrowth in PC12 cells.

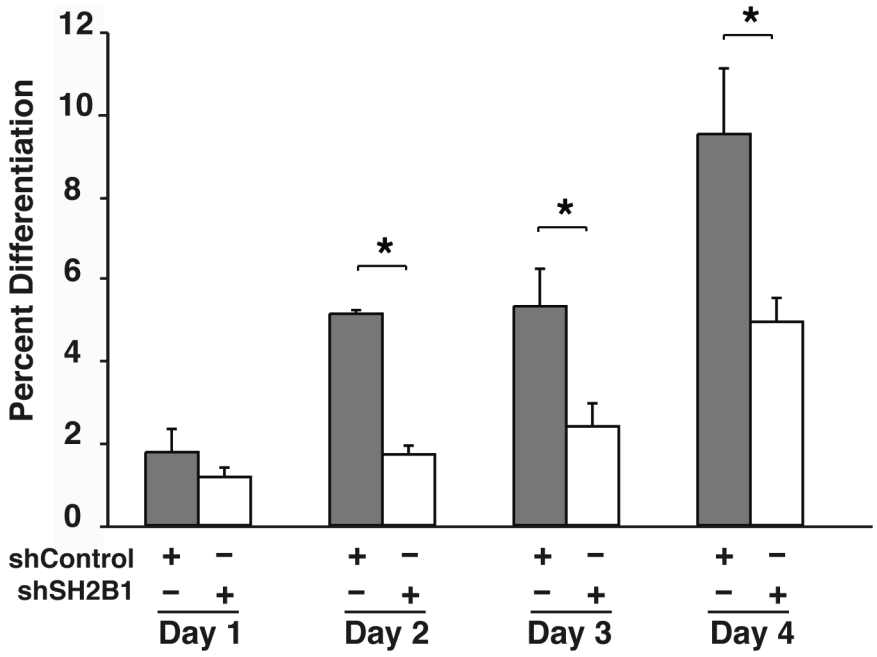


FIG. 4.1. Endogenous SH2B1 is required for NGF-dependent neurite outgrowth.

PC12 cells stably expressing control shRNA (shControl) or shRNA targeted against SH2B1 (shSH2B1) were incubated in serum-free medium for 14 h and then treated with 50 ng/ml of NGF for 4 days. The percentage of differentiated cells (y-axis) was calculated on days 1, 2, 3, and 4 of the 4-day assay (x-axis). The values are means ± standard error of the mean from three different experiments. Asterisks represent p values of <0.05 using a one-tailed, paired Student T-test. At least 250 cells were counted for each condition.

Next, we wanted to address the critical question of whether or not endogenous SH2B1 is required for NGF-dependent activation of ERKs 1 and 2, since ERK activation is critical for NGF-induced neurite outgrowth of PC12 cells (28-30). Both shControl and shSH2B1 PC12 cells were treated with NGF (50 ng/ml) for 0, 15, 120, 240, and 360 min. As mentioned above, FIG. 4.2, top panel, reveals a ~50% reduction of endogenous SH2B1β in the shSH2B1 versus shControl cells. Additionally, this panel reveals that the mobility of endogenous SH2B1β is dramatically reduced after 15 min of treatment with NGF, as a result of its extensive tyrosyl and serine/threonine phosphorylation, as reported previously (68). In order to assess the NGF-dependent activation of ERKs 1 and 2, we blotted the membrane with αpERK which recognizes only the doubly phosphorylated, activated form of ERKs 1 and 2 as well as an antibody against total ERK (αERK). As shown in FIG. 4.2, middle panel, the extent and duration of phosphorylation of ERKs 1 and 2 in response to NGF was similar in the shControl and shSH2B1 cell lines. The membrane was reblotted with an antibody to pAkt (αpAkt) that recognizes phosphorylated Ser 473. Akt, which lies downstream of PI3 kinase, requires phosphorylation on Ser 473 to be active (246). Similarly, we observed no significant difference in phosphorylated Akt levels in the shSH2B1 line compared to the shControl line. These results suggest that SH2B1 is not required for NGF activation of Akt or ERKs 1 and 2.

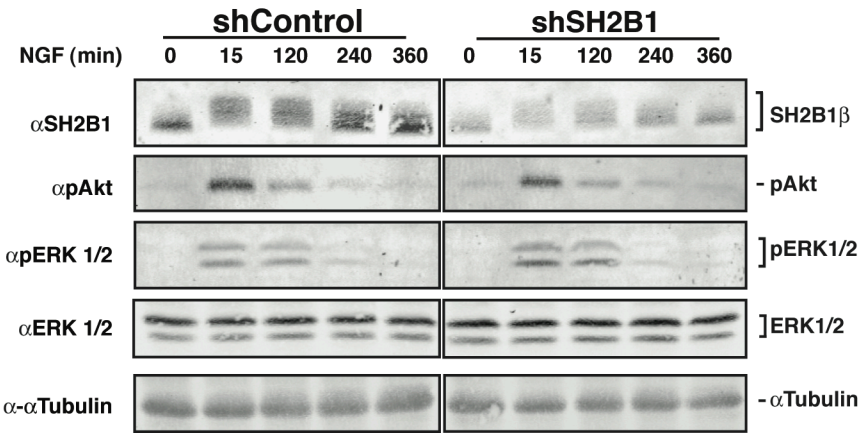


FIG. 4.2. Effect of shRNA-mediated knockdown of endogenous SH2B1 on NGF-induced phosphorylation of Akt and ERKs 1 and 2.

PC12 cells stably expressing control shRNA (shControl) or shRNA targeted against SH2B1 (shSH2B1) were incubated in serum-free medium for 14 h before treatment with 50 ng/ml NGF for 0, 15, 120, 240, or 360 min. Proteins in cell lysates were separated by SDS-PAGE and immunoblotted with (A) αSH2B1, (B) αpAkt(Ser473), (C) αpERK, (D) αErk, and (E) α-αTubulin as a loading control.

Identification of a region within SH2B1 required for nuclear translocation.

We have shown previously (79) that deletion of amino acids 198-268 results in the nuclear accumulation of SH2B1β, an otherwise membrane/cytoplasmic localized protein (33). Close examination of the sequence contained within amino acids 198-268 revealed a match (79) to the nuclear export sequence (NES) motif ($LX_{2-3}LX_{2-4}LXL$) (118, 247, 248). Indeed, point mutations of two key leucine residues (L231 and 233) in this sequence rendered SH2B1β defective in nuclear export, resulting in SH2B1β's nuclear accumulation (79). The dramatic localization shift observed with these SH2B1β mutants suggested that SH2B1β undergoes rapid and continual shuttling between the nucleus and cytoplasm, with the rate of nuclear export greatly exceeding the rate of its nuclear import, giving rise to a steady-state membrane/cytoplasmic appearance. While we had shown that SH2B1β contained a functional NES, it was still unclear how exactly SH2B1β localized to the nucleus in the first place. To address this, the dramatic nuclear accumulation of GFP-SH2B1β(Δ198-268) lacking the NES (henceforth designated as GFP-SH2B1β(ΔNES)) (FIG. 4.3A, b) or GFP-SH2B1β(WT) treated with the nuclear export inhibitor leptomycin B (LMB) (FIG. 4.3A, a), was used as the readout of a screen to identify the region(s) necessary for SH2B1β's nuclear import. In this screen in which nuclear export is blocked, nuclear accumulation of SH2B1β represents functional nuclear import of SH2B1β whereas nuclear exclusion represents defective nuclear import of SH2B1β. Initially, a series of truncation, deletion, and point mutations were made on top of GFP-SH2B1β(ΔNES) and transiently expressed in COS-7 cells. COS-7 cells were used for the initial screen because of their high transfectional efficiency and flat morphology which lends itself to rapid and unambiguous identification of subcellular localization of the fluorescent proteins. Regions in SH2B1β(ΔNES) that contained mutations or deletions that resulted in the failure of GFP-SH2B1β(ΔNES) to accumulate in the nucleus were considered implicit for nuclear import. We made the same series of

mutations in a GFP-tagged, wild-type SH2B1β and incubated the cells expressing those mutants in the absence or presence of LMB.

As mentioned previously, overexpressed GFP-SH2B1β(WT) appears to have a membrane and cytoplasmic localization (FIG. 4.3A, a; left panel). After incubation with LMB, GFP-SH2B1β(WT) was found to accumulate in the nucleus (FIG. 4.3A, a; right panel), as shown previously (79). The GFP-SH2B1β(ΔNES) mutant retains the ability to translocate to the nucleus, but is unable to cycle to the cytoplasm without the sequence responsible for nuclear export (FIG. 4.3A, b). Another mutant of SH2B1β [SH2B1β(Δ147-268)] which contains a slightly larger region of deletion than SH2B1β(ΔNES) (Δ198-268) was observed in the cytoplasm with or without LMB (FIG. 4.3A, c). This suggests that the region required for nuclear import of SH2B1 lies within amino acids 147-198. As predicted, GFP-SH2B1β(Δ147-198) failed to accumulate in the nuclei of COS-7 cells in the absence or presence of LMB (FIG. 4.3A, d). Examination of the amino acid sequence between 147 and 198 in SH2B1β (FIG. 4.3B) revealed a small stretch of highly basic residues that resembles the classical basic amino acid motif (PKKKRKV) of the SV40 large T antigen (249, 250). This putative nuclear localization sequence is almost perfectly conserved in all known vertebrate SH2B1 homologues from zebrafish to human (FIG. 4.3B). The basic region identified in SH2B1 spans from amino acid 146-152, thus four basic residues likely to be critical for nuclear import were missing from the nuclear import defective SH2B1β(Δ147-198).

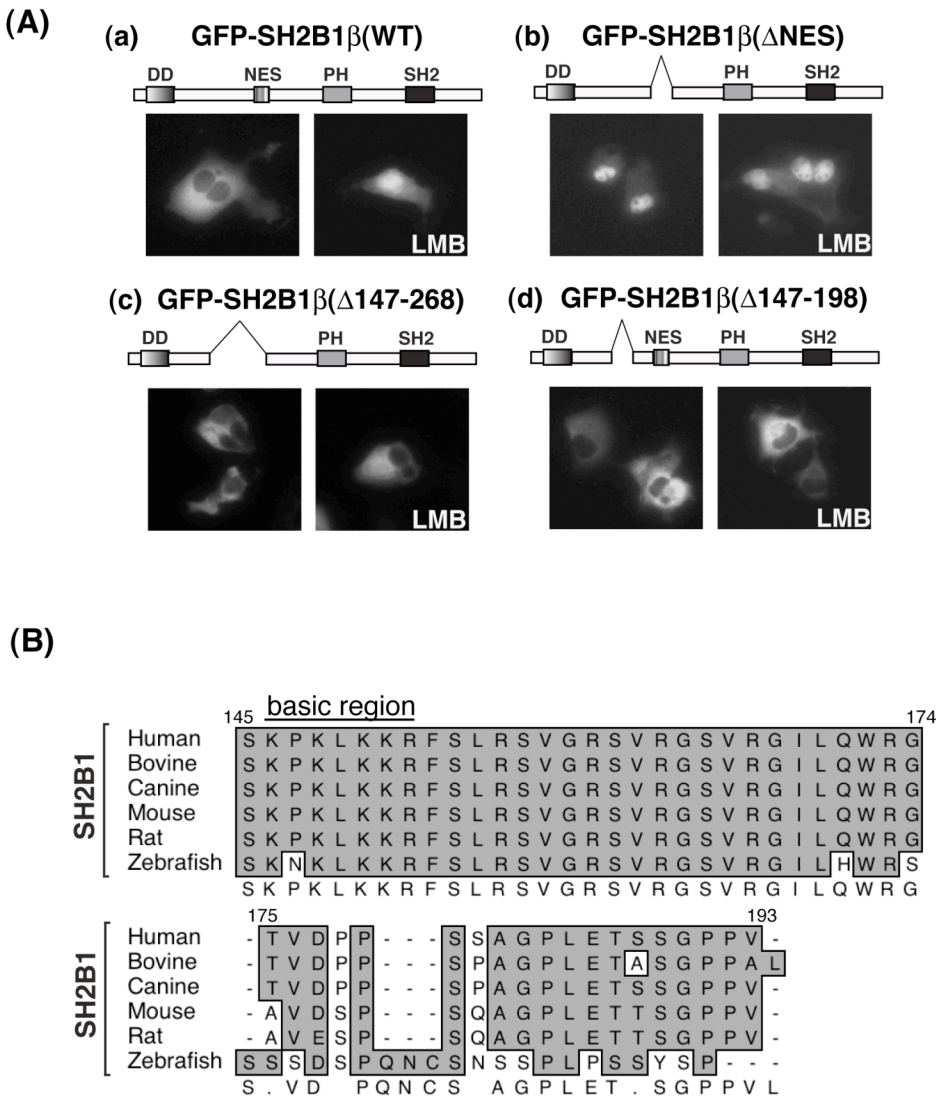


FIG. 4.3. The region between amino acids 147-198 is essential for nuclear translocation.

(A) COS-7 cells were transiently transfected with cDNA encoding GFP-SH2B1β(WT) (a), GFP-SH2B1β(ΔNES) (b), GFP-SH2B1β(Δ147-268) (c), or GFP-SH2B1β(Δ147-198) (d). Fourteen hours post transfection, the cells were either mock treated with vehicle (left panels) or treated with 20 nM leptomycin B (LMB) (right panels) for 7 h. Cells were then fixed and visualized using epifluorescence microscopy. The schematic representations of SH2B1β indicate the positions of internal deletions and/or previously defined domains within SH2B1, including the dimerization domain (DD), nuclear export sequence (NES), pleckstrin homology domain (PH), and Src homology domain (SH2). (B) MacVector ClustalW sequence alignment of multiple species of SH2B1 between residues 145 and 193.

SH2B1 requires an NLS and a functional SH2 domain for nuclear translocation.

To confirm that this basic region found between amino acids 146-152 is responsible for nuclear import of SH2B1β in PC12 cells, a series of GFP-SH2B1β constructs encoding point mutations and/or deletions in this region were expressed in PC12 cells in the presence or absence of LMB. As shown above in FIG. 4.3A, GFP-SH2B1β(WT) appears to localize exclusively to the membrane and cytoplasm (FIG. 4.4A, top left panel). Incubation with LMB, however, results in the accumulation of GFP-SH2B1β(WT) in the PC12 nucleus (FIG. 4.4A, top right panel). Similarly as shown above in FIG. 4.3A, b, the localization of GFP-SH2B1β(ΔNES) was found to be nuclear whether or not it was incubated with LMB (FIG. 4.4B). We used site directed mutagenesis to change the putative NLS from K148/K150/K151/R152 to A148/A150/A151/A152 in SH2B1β. This mutant SH2B1β will be referred to as SH2B1β(mNLS). The mutation of the putative NLS resulted in a failure of GFP-SH2B1β to localize to the nucleus even in the presence of LMB (FIG. 4.4C) or when combined with deletion of the NES (FIG. 4.4D). We therefore concluded that the basic residues K148/K150/K151/R152 are required for the nuclear localization of SH2B1β and constitute a previously unrecognized monopartite NLS present within all isoforms of SH2B1.

During our attempts to find the region required for SH2B1β nuclear import, we found that GFP-SH2B1β(R555E) displayed a significantly reduced, or possibly, a complete inability to translocate to the nucleus. FIGS. 4.4E and 4.4F illustrate that GFP-SH2B1β(R555E) does not accumulate in the nucleus when treated with LMB (FIG. 4.4E) or when the NES is deleted (FIG. 4.4F). As described previously (39), the mutation of the critical arginine within the SH2 domain to a glutamate (SH2B1β(R555E)) abolishes the phospho-tyrosine binding ability of SH2B1, and the expression of SH2B1β(R555E) in PC12 cells results in the “dominant negative” inhibition of NGF-dependent differentiation.

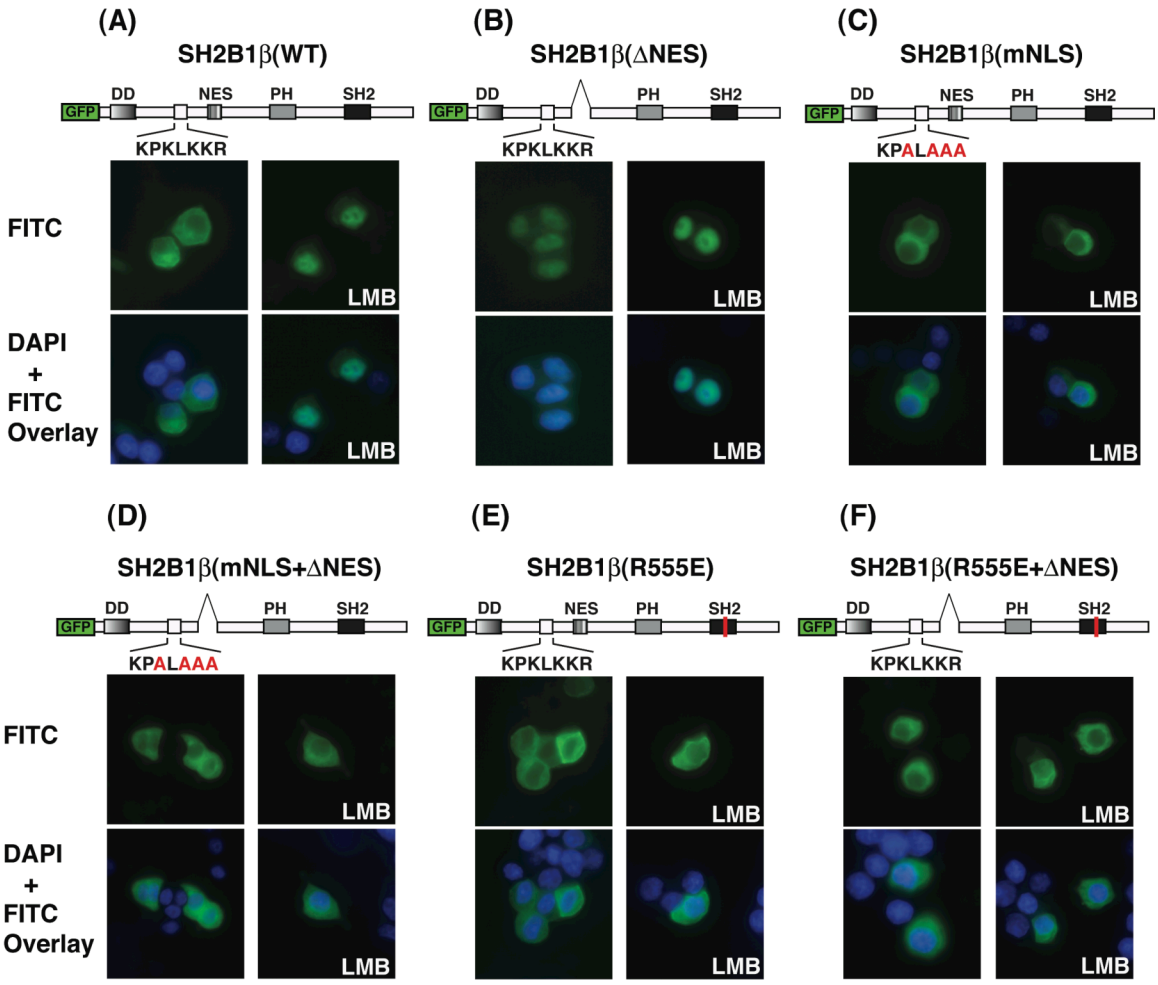


FIG. 4.4. Point mutations in the putative NLS motif inhibit nuclear accumulation of SH2B1β.

PC12 cells were transiently transfected with cDNAs encoding GFP-SH2B1β(WT) (A), GFP-SH2B1β(ΔNES) (B), GFP-SH2B1β(mNLS) (C), GFP-SH2B1β(mNLS+ΔNES) (D), GFP-SH2B1β(R555E) (E), and GFP-SH2B1β(R555E+ΔNES) (F). Fourteen h post transfection, the cells were either mock treated (left panels) or treated with 20 nM leptomycin B (LMB) for 7 h (right panels). Cells were then fixed and stained with DAPI to visualize nuclei (blue color in overlay image, lower panels) and then visualized using epifluorescence microscopy. The schematic representations of SH2B1β indicate the positions of internal deletions and/or previously defined domains within SH2B1, including the dimerization domain (DD), nuclear export sequence (NES), pleckstrin homology domain (PH), and Src homology domain (SH2). The open white box represents the putative NLS motif within SH2B1 and the corresponding sequence is indicated below. Single basic amino acid substitutions to alanines within the NLS are indicated in red.

SH2B2 does not undergo nucleocytoplasmic shuttling

The finding that SH2B1 contains functional nuclear export and localization signals raises the question as to whether other SH2B gene family members [SH2B2 (APS), SH2B3 (Lnk)] can also undergo nucleocytoplasmic shuttling. The ClustalW multiple sequence alignment of several species of SH2B1 family members revealed that SH2B2, but not SH2B3, shares a high degree of homology in both the nuclear localization (FIG. 4.5A) and nuclear export sequences (FIG. 4.5B) of SH2B1. The alignment also revealed that three of the four basic residues required for SH2B1 nuclear localization (indicated with arrows) are conserved in rat, mouse, and human SH2B2 (R119, R121, and K122). Further, SH2B2 contains a putative NES between the residues 168 and 174, based on the loosely defined NES motif, LX2-3LX2-4LXL (118, 247, 248). The putative NES of SH2B2 contains three of the four hydrophobic residues, including the amino acid motif LXL (FIG. 4.4B), that has been shown to be required for nuclear export of SH2B1 (79) as well as of 14-3-3 (118).

In agreement with previous findings (63, 251), SH2B2(WT) appears to be localized at the plasma membrane as well as the cytoplasm, as shown in FIG. 4.5C, a. The subcellular localization of myc-SH2B2 resembled closely the localization of GFP-SH2B1β (FIG. 4.5C, b). However, while GFP-SH2B1β(ΔNES) is localized to the nucleus (FIG. 4.5B, c), myc-tagged SH2B2 mutant carrying a deletion of the putative NES (Δ167-184) [SH2B2(ΔNES)] did not accumulate in the nucleus (FIG. 4.5C, d). To confirm the inability of SH2B2 to undergo nucleocytoplasmic shuttling, we expressed myc-tagged SH2B2(WT) and incubated the cells in the presence of LMB for 24 h. As shown in FIG. 4.5C, e, LMB treatment did not cause the nuclear accumulation of myc-SH2B2(WT). Mutations to the putative NLS (K117A, R119A, R121A, and K122A) [SH2B2(mNLS)] also did not alter the subcellular localization of SH2B2 (FIG. 4.5C, f). To address the possibility that SH2B2 translocates to the nucleus

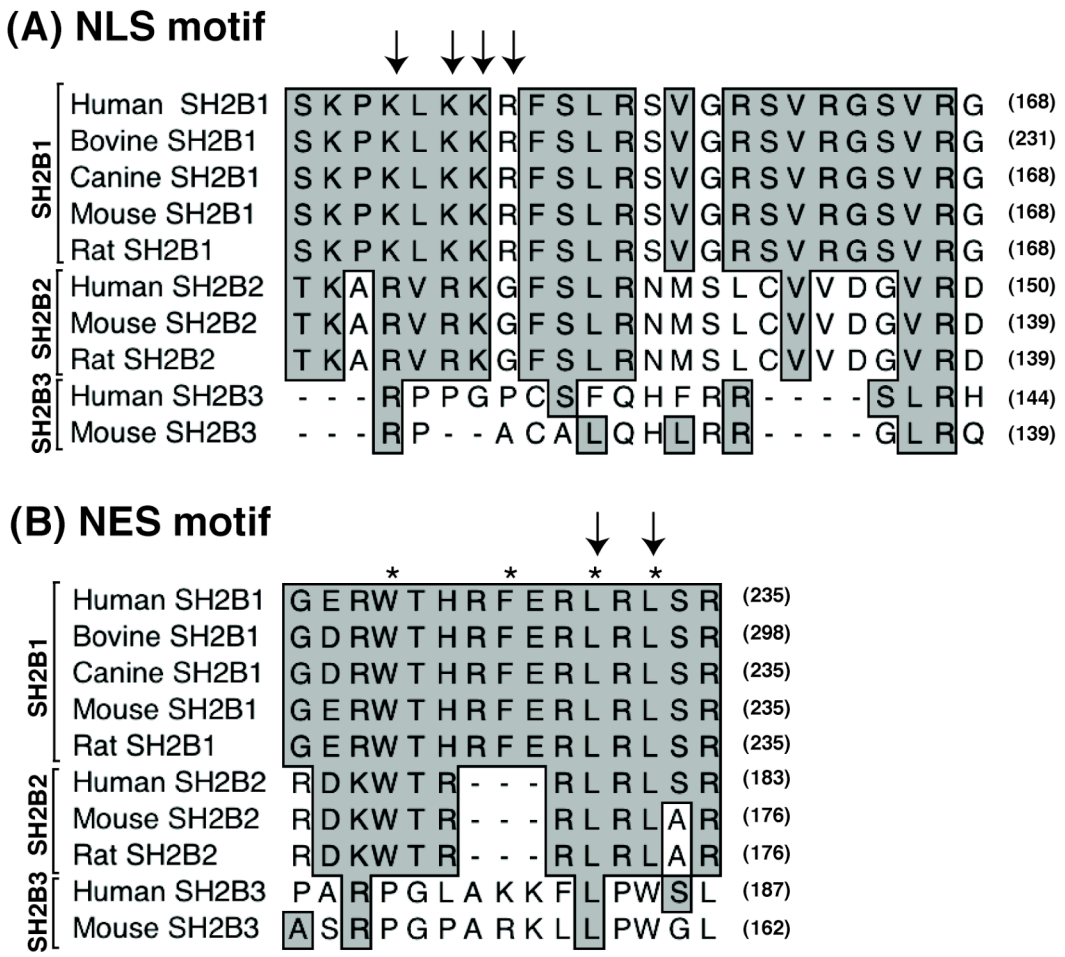


FIG. 4.5A, B. Subcellular distribution of internal deletion and point mutants of SH2B2 in PC12 cells.

MacVector ClustalW multiple sequence alignment was used to compare several species of SH2B1, SH2B2, and SH2B3. (A) Inter-species conservation among SH2B family members within the region corresponding to the NLS of SH2B1. Arrows designate the basic residues required for nuclear import. (B) Inter-species conservation among SH2B family members within the region corresponding to the NES of SH2B1. Asterisks denote the general NES motif and arrows designate the hydrophobic residues that have been shown to be required for SH2B1β nuclear export.

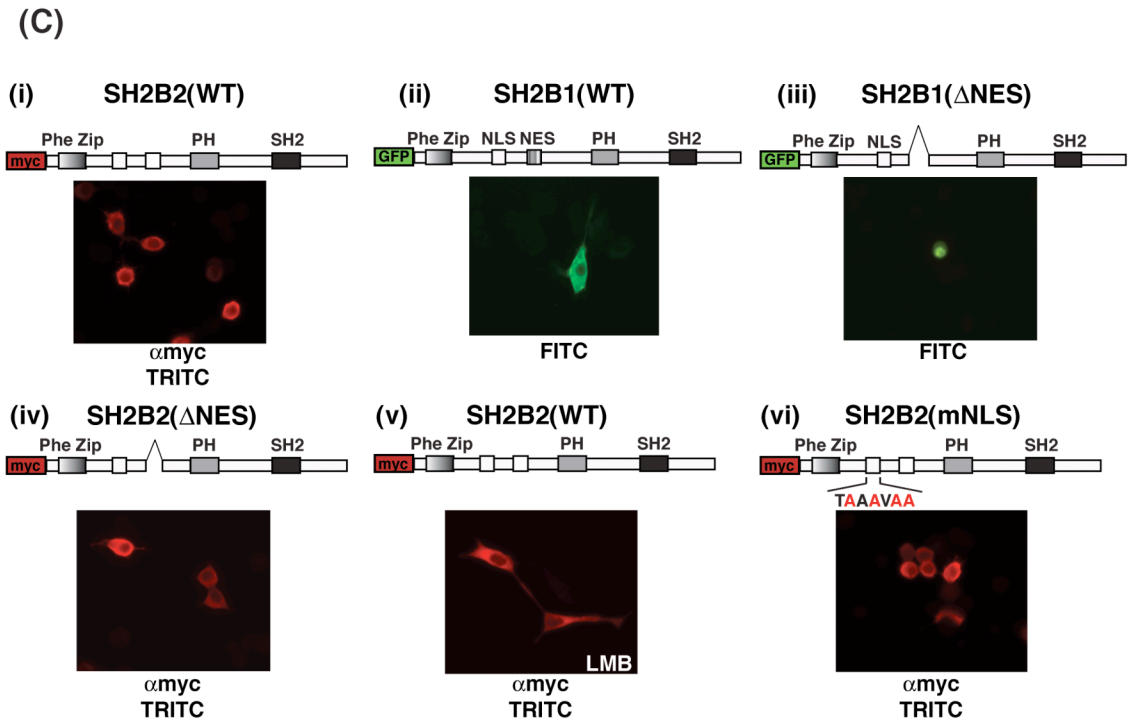


FIG. 4.5C. Subcellular distribution of internal deletion and point mutants of SH2B2 in PC12 cells.

(C) PC12 cells were transiently transfected with cDNAs encoding (i) myc-SH2B2(WT), (ii) GFP-SH2B1β(WT), (iii) GFP-SH2B1β(ΔNES), (iv) myc-SH2B2(ΔNES), (v) myc-SH2B2(WT), and (vi) myc-SH2B2(mNLS). Fourteen hours post transfection, the cells were either treated with vehicle (methanol) (panels i, ii, iii, iv, and vi) or treated with 20 nM leptomycin B (LMB) (panel v) for 7 h. Cells transiently expressing myc-tagged constructs were stained with αmyc and anti-mouse IgG Alexa 594 and are visualized in the TRITC channel (panels i, iv, v, and vi). GFP-SH2B1 expression is visualized in the FITC channel (panels ii and iii). Images were taken using epifluorescence microscopy.

only in response to NGF stimulation or possibly serum deprivation, myc-SH2B2(ΔNES) expressing cells were incubated in serum-free medium for 14 h and incubated in the absence or presence of NGF for 5 min, 30 min, 1 h, and 6 h. None of the conditions tested resulted in nuclear accumulation of myc-SH2B2(ΔNES) (data not shown). The failure to detect even the slightest nuclear localization of SH2B2 after inhibiting nuclear export with LMB or removing its putative NES, suggests that the cytoplasmic appearance of SH2B2 reflects a more-or-less static localization. This is in contrast to SH2B1β(WT) which is seemingly in a constitutive flux between the plasma membrane/cytoplasm and nucleus. Consequently, we have concluded that in spite of the high degree of sequence similarity between family members SH2B1 and SH2B2, the ability to undergo nucleocytoplasmic shuttling is unique to SH2B1.

Ability of nuclear localization defective SH2B1β mutants to regulate NGF-induced differentiation.

To determine whether the nuclear localization of SH2B1β is required for NGF-dependent differentiation of PC12 cells, we tested the effects of the nuclear localization sequence deficient SH2B1β mutant, SH2B1β(mNLS), on NGF-induced neurite outgrowth. PC12 cells stably expressing vector control, SH2B1β(WT), SH2B1β(R555E) or SH2B1β(mNLS) were incubated in differentiation medium in the absence or presence of 50 ng/ml of NGF for a total of 5 days (FIG. 4.6A). In agreement with previous findings, stable overexpression of SH2B1β(WT) resulted in more than a doubling of the number of cells exhibiting NGF-dependent neurite outgrowth (38% compared to 18% for control cells) (FIG. 4.6B). Likewise, the stable expression of the dominant negative form of SH2B1β, SH2B1β(R555E), resulted in inhibition of NGF-dependent neurite outgrowth (13% of cells) compared to GFP alone (18% of cells) (FIG. 4.6B). Strikingly, as shown in FIGS. 4.6A and 4.6B, expression of SH2B1β(mNLS) inhibited NGF-dependent neurite outgrowth to the same extent

as expression of SH2B1β(R555E) (13%). The cell lines were created by pooling together the top 5% GFP positive cells, using FACS sorting after 4 weeks of selection in G418 containing growth medium. The resulting stable PC12 cell lines display a spectrum of GFP expression levels and in FIG. 4.6A, only PC12 cells expressing "high" levels of GFP can be seen after merging the fluorescent and bright-field images, although all cells are GFP positive and were counted as such. The inhibition of NGF-dependent differentiation resulting from GFP-SH2B1β(mNLS) is even more evident when the "high" GFP-SH2B1β(WT) expressing cells which exhibit enhanced neurite length, are compared to the growth stunted neurites of the "high" GFP-SH2B1β(mNLS) expressing cells.

Effect of overexpression of SH2B1β(mNLS) on NGF-induced tyrosyl phosphorylation of TrkA and activation of ERKs 1 and 2

In PC12 cells, prolonged activation of ERK is thought to mediate differentiation, whereas transient activation of ERK is thought to promote proliferation (27). Because overexpression of SH2B1β(mNLS) inhibits NGF-dependent neurite outgrowth, it was of interest to establish whether the overexpression of SH2B1β(mNLS) affects the kinetics of NGF-dependent activation of TrkA and ERKs 1 and 2. PC12 cell lines stably expressing GFP, GFP-SH2B1β(WT), or GFP-SH2B1β(mNLS) were incubated in serum free-medium and then stimulated with NGF for 0, 15, 120, 240, and 360 min. Proteins from cell lysates were resolved by SDS-PAGE and immunoblotted first with αEGFP to verify the presence of GFP-SH2B1β(WT) and GFP-SH2B1β(mNLS). Importantly, FIG. 4.7 reveals that expression levels of GFP-SH2B1β(WT) and GFP-SH2B1β(mNLS) are similar, therefore eliminating the potential for SH2B1β dosage dependent differences. Figure 7 also reveals

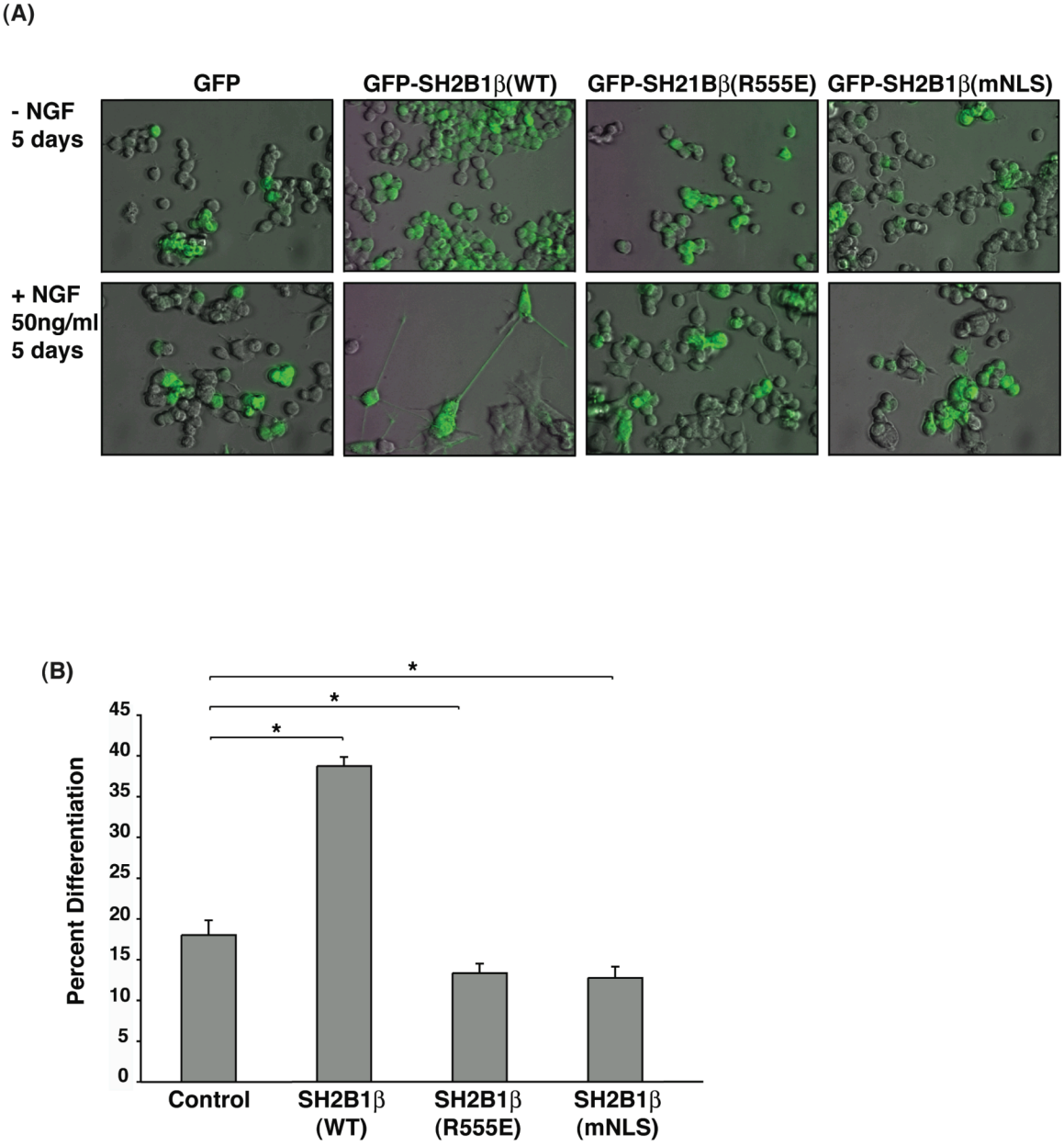


FIG 4.6. Stable expression of the SH2B1β nuclear import mutant inhibits NGF-dependent neurite outgrowth of PC12 cells.

(A) PC12 cells stably expressing GFP, GFP-SH2B1β(WT), GFP-SH2B1β(R555E), or GFP-SH2B1B(mNLS) were incubated in serum-free medium overnight before incubation in the absence (top panels) or presence (bottom panels) of 50 ng/ml NGF for 5 days. Representative pictures of each condition were imaged by overlaying the FITC channel (GFP) on top of standard bright field illumination. (B) The percentage of differentiated cells was scored on day 5. Means ± standard errors of the mean from 6 different experiments are shown. Asterisks represent p values of <0.05 using a one-tailed, paired Student T-test. A combined total of at least 400 cells were counted in each condition.

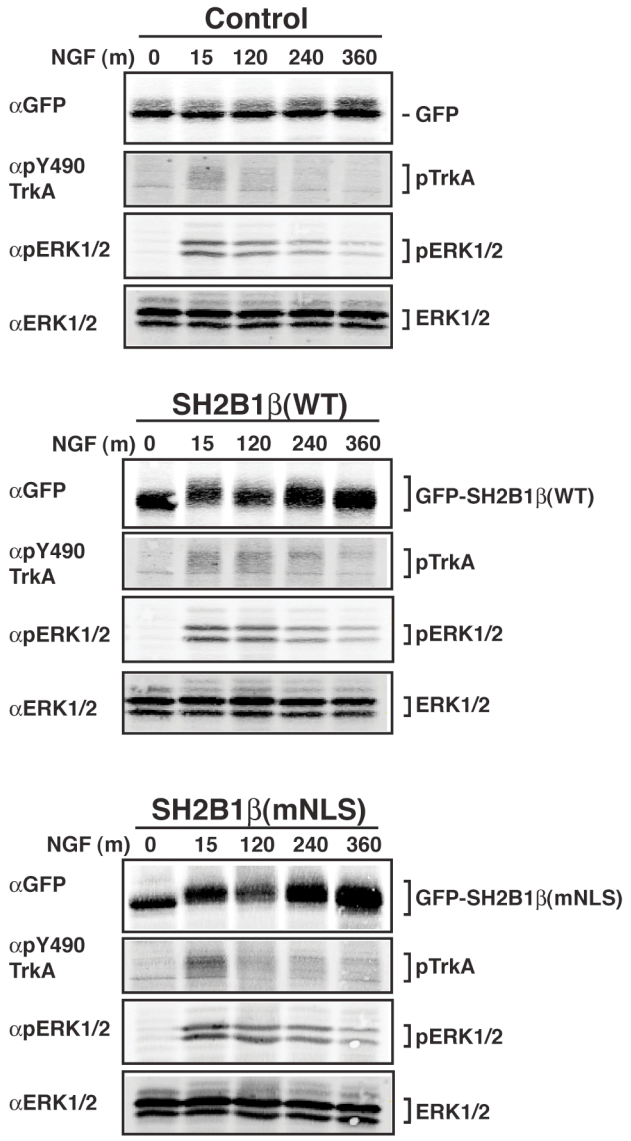


FIG. 4.7. Effects of SH2B1β(mNLS) expression on NGF-induced phosphorylation of TrkA and ERKs 1 and 2.

PC12 cells stably expressing GFP, GFP-SH2B1β(WT), or GFP-SH2B1β(mNLS) were incubated in serum-free medium overnight before treatment with 50 ng/ml NGF for 0, 15, 120, 240, and 360 min. Proteins in cell lysates were separated by SDS-PAGE and immunoblotted with αGFP to visualize the relative levels of expression of GFP-SH2B1β(WT) and GFP-SH2B1β(mNLS). The blots were also immunoblotted with αpTrkA(Y490), αpERK1/2, and αERK1/2.

similar upward shifts in migration of GFP-SH2B1β(WT) and GFP-SH2B1β(mNLS), consistent with the 2 forms of SH2B1β undergoing similar degrees of phosphorylation in response to NGF. Immunoblotting with an antibody that recognizes phosphotyrosine 490 (αpY490 TrkA), a primary autophosphorylation site within TrkA, revealed a similar NGF-dependent phosphorylation of TrkA in the SH2B1β(WT) and SH2B1β(mNLS) expressing cell lines. Likewise, the extent of phosphorylation of ERKs 1 and 2 after NGF treatment, as well as the duration of phosphorylation, was comparable between SH2B1β(WT) and SH2B1β(mNLS) expressing cell lines. Collectively, these data strongly suggest that the neurite outgrowth defect observed in the cells expressing SH2B1β(mNLS) is not secondary to a defect in NGF-dependent activation of TrkA or ERKs 1 and 2.

Nuclear import and export of SH2B1β are required for NGF-induced enhancement of gene expression required for neuronal differentiation

In a recent publication (124), we reported that SH2B1β enhances NGF-dependent neurite outgrowth in part by amplifying the expression of a specific subset of NGF-responsive genes. The genes identified included uPAR, Mmp3, and Mmp10. These proteins were of particular interest because uPAR has been shown to be required for NGF-mediated neuronal differentiation (122) and MMP3/10 are both situated in the same proteolytic cascade as uPAR, responsible for extracellular matrix degradation (reviewed in References (191) and (192)). Consistent with our finding that endogenous SH2B1β is required for NGF-dependent neurite outgrowth (FIG. 4.2), shRNA mediated knockdown of SH2B1β suppressed the NGF-dependent induction of uPAR, Mmp3, and Mmp10 (124). To test the hypothesis that nuclear translocation of SH2B1β facilitates the transcription of genes required for NGF-induced neuronal differentiation, we used QT-PCR to assay the NGF-dependent transcription of uPAR, Mmp3, and Mmp10. PC12 cells stably expressing vector control, SH2B1β(WT),

SH2B1β(R555E) and SH2B1β(mNLS) were incubated in serum-free medium for 14 h and then incubated with or without NGF (100 ng/ml) for 0 or 6 h. Untreated PC12 cells displayed very little basal transcription of uPAR, Mmp3, or Mmp10 (FIG. 4.8). After 6 h of NGF treatment, transcription of these genes was dramatically elevated. In agreement with our previous findings (124), NGF-induced transcription of uPAR, Mmp3, and Mmp10 was enhanced in cells stably expressing SH2B1β(WT), and reduced in cells stably expressing SH2B1β(R555E) (FIG. 4.8 A-C). Overexpression of SH2B1β(mNLS) failed to enhance NGF-induced transcription of uPAR, Mmp3, or Mmp10, as shown in FIGS. 4.8A, B, and C, respectively. The inability of SH2B1β(mNLS) to enhance transcription of uPAR, Mmp3, or Mmp10, implies that the nuclear translocation of SH2B1β is critical for SH2B1β enhancement of NGF-induced transcription of these genes. The aforementioned results indicate that SH2B1β has the capacity to translocate into the nucleus and enhance the transcription of specific NGF-responsive genes. Although SH2B1β does not contain any presently discernable DNA binding or transactivation domains, the findings raise the possibility that SH2B1β may be acting as a transcription factor or co-activator for these genes. If this were the case, expression of the constitutively nuclear localized SH2B1β(ΔNES) mutant might enhance the transcription of these genes over that of cells expressing SH2B1β(WT). To determine the effects of expressing SH2B1β(ΔNES), we compared the NGF-dependent transcription of Plaur, Mmp3, and Mmp10 in the same stable lines control, SH2B1β(WT), and SH2B1β(ΔNES). Again, the cells were serum deprived for 14h before treatment with 100ng/ml of NGF for 0 and 6h. Consistent with our previous results, the expression of SH2B1β(WT) enhanced, while the expression of SH2B1β(R555E) inhibited, NGF-induced transcription of Plaur, Mmp3, and Mmp10 without affecting the transcription of GAPDH (FIG. 4.9). We observed a modest, but not statistically significant, increase in the NGF-dependent transcription of both Plaur

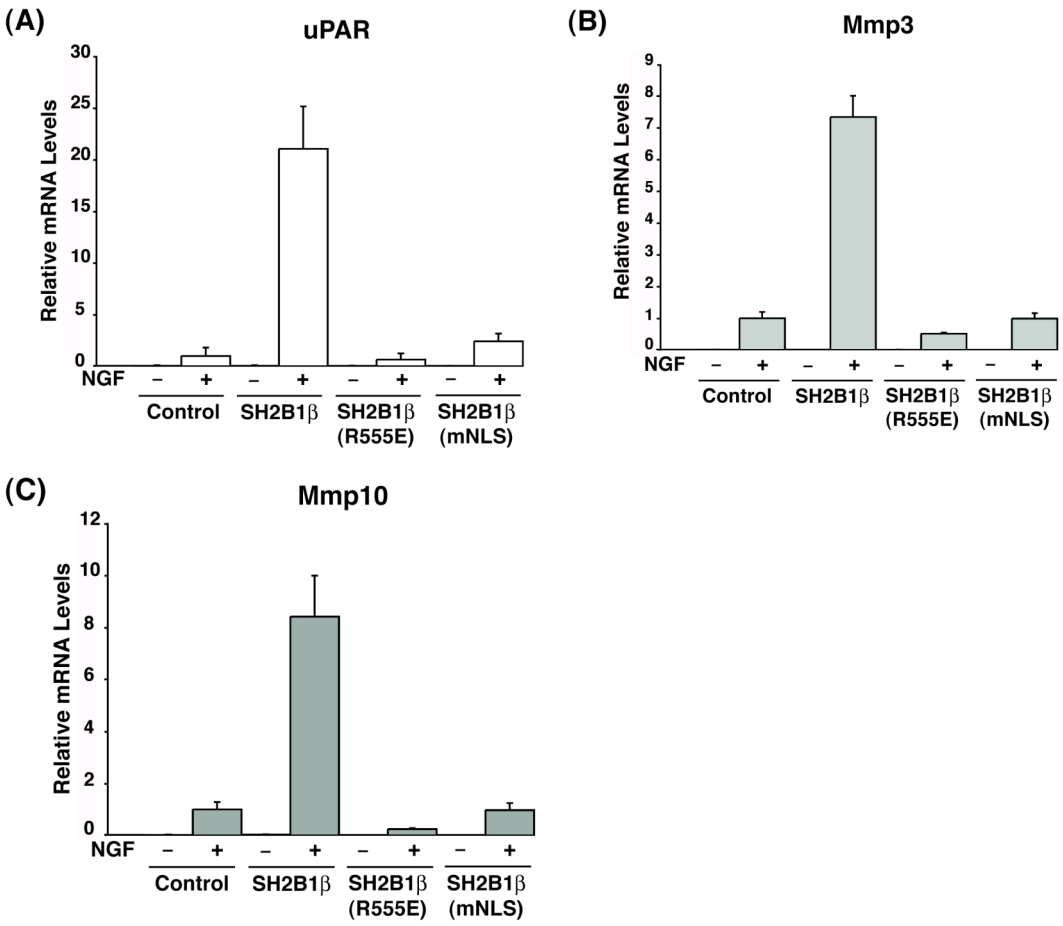


FIG. 4.8. Stable expression of the SH2B1β nuclear import mutant (SH2B1β(mNLS)) fails to enhance NGF-induced transcription of uPAR, Mmp3, and Mmp10.

After incubation in serum-free medium overnight, PC12 cells stably expressing GFP, GFP-SH2B1β(WT), GFP-SH2B1β(R555E), or GFP-SH2B1B(mNLS) were incubated with or without 50 ng/ml NGF for 6h. The NGF-dependent induction of mRNA for (A) Plaur (uPAR), (B) Mmp3, (C) and Mmp10 was assessed using QT-PCR. Target gene expression was normalized first to levels of GAPDH and then to levels of gene expression seen in GFP control cells treated with NGF. Means ± standard errors of the mean from 4-6 separate experiments are shown.

(FIG. 4.9A) and Mmp10 (FIG. 4.9C) in the SH2B1β(ΔNES) expressing cells compared to control. However, the NGF-dependent transcription of Plaur, Mmp3, and Mmp10 in the SH2B1β(ΔNES) cells was significantly reduced compared to SH2B1β(WT) cells. These findings demonstrate that constitutively nuclear localized SH2B1β is significantly less effective than SH2B1β(WT) at enhancing NGF-dependent transcription of genes required for neurite outgrowth. Collectively, the QT-PCR data indicate that SH2B1β requires both a functional NLS and NES to enhance NGF-dependent transcription of Plaur, Mmp3, and Mmp10.

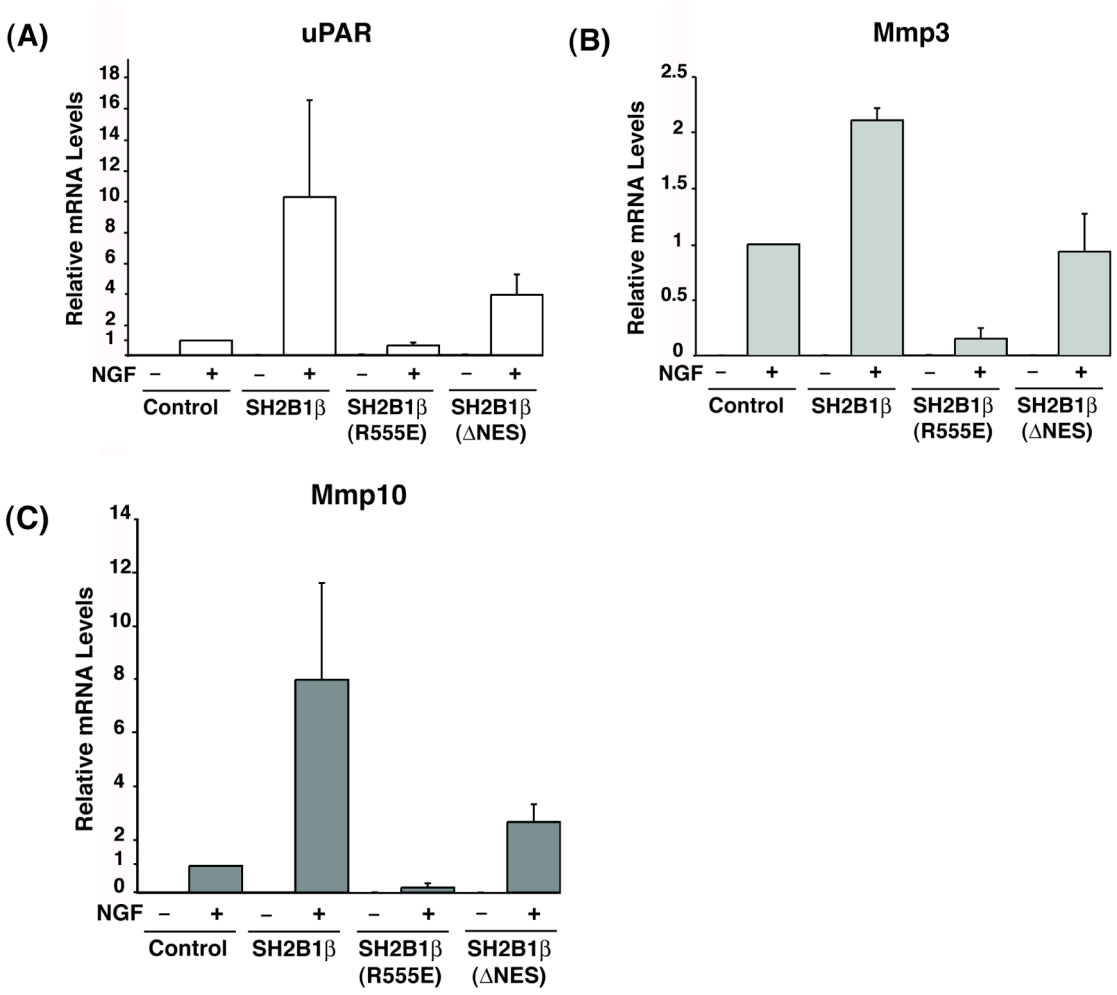


FIG. 4.9. Stable expression of the SH2B1β nuclear export mutant (SH2B1β(ΔNES)) fails to enhance NGF-induced transcription of uPAR, Mmp3, and Mmp10.

PC12 cells stably expressing GFP, GFP-SH2B1β(WT), GFP-SH2B1β(R555E), or GFP-SH2B1B(ΔNES) were incubated in serum-free medium overnight and then incubated with or without 50 ng/ml NGF for 6h. The NGF-dependent induction of mRNA for (A) uPAR, (B) Mmp3, and (C) Mmp10 was quantified using QT-PCR. Target gene expression was normalized first to levels of GAPDH and then to levels of gene expression seen in GFP control cells treated with NGF. Means and range are shown for 2 separate experiments.

Discussion

In this work, we investigated the mechanism by which SH2B1β enhances NGF-dependent neurite outgrowth in PC12 cells. We report here that SH2B1β is required for NGF-dependent neurite outgrowth in a manner that is distinct from the activation of the PI3K/Akt and/or Ras-ERK pathways. Previous studies have attempted to answer the question of whether SH2B1β is required for neurite outgrowth, but have relied upon the overexpression of dominant negative mutants of SH2B1 (33, 96). Here, we have addressed this question more directly by showing that when endogenous levels of SH2B1 are reduced in PC12 cells using shRNA targeted against all isoforms of SH2B1, NGF-dependent differentiation is inhibited proportionate to the extent of SH2B1 knockdown. Importantly, the knockdown of SH2B1 did not alter the extent and/or duration of NGF-induced phosphorylation of Akt or ERKs 1 and 2. Recent work regarding a highly analogous signaling system to that of NGF/TrkA (35) demonstrated that SH2B1 is also required for glial-cell-line-derived neurotrophic factor (GDNF)-dependent neurite outgrowth (35). The inhibition of GDNF-dependent neurite outgrowth that resulted from either the overexpression of the dominant negative SH2B1β(R555E) or SH2B1β RNAi was similarly not a consequence of reduced signaling, as the respective levels of GDNF-induced phosphorylation of Akt and ERKs 1 and 2 were the same as in control cells (35). Collectively, these data strongly suggest that endogenous SH2B1 is critical for NGF-induced neuronal differentiation and enhances NGF-induced differentiation at a point downstream of or parallel to ERKs 1 and 2.

Consistent with the idea that SH2B1β may function downstream or parallel to ERK1/2 activation, we show that nuclear SH2B1β is required for NGF-dependent differentiation of PC12 cells. In addition to the previously described NES, we have identified within SH2B1 a functional nuclear localization sequence contained within a stretch of basic amino acids (a.a. 146-152). Internal deletion

of these amino acids blocked SH2B1β accumulation in the nucleus, even when nuclear export was blocked by the deletion of the NES or the presence of LMB. Specific mutation of four basic amino acids to alanines within the putative NLS [SH2B1β(mNLS)] blocked SH2B1β nuclear translocation in PC12 cells and all other cell lines tested (293T, COS-7, and NIH 3T3, data not shown). The nuclear localization sequence is almost perfectly conserved in all SH2B1 isoforms as well as all known vertebrate SH2B1 homologues from human to zebrafish, suggesting that the NLS bestows an important function to the SH2B1 protein.

Based upon the high degree of sequence similarity between SH2B1 and SH2B2, we were somewhat surprised to find that SH2B2 did not also cycle between the cytoplasm and nucleus. After the initial identification of SH2B1 and SH2B2 in neurons (34), early conclusions drawn from their characterization indicated a large amount of functional overlap between the two proteins. SH2B1 and SH2B2 were both shown to associate with activated TrkA as well as enhance NGF-dependent neuritogenesis (33, 34). In recent years, however, functionally distinct differences between SH2B1 and SH2B2 have been observed, including structural differences within their SH2 domains, which account for phosphotyrosine binding site specificity (252), and the identification of unique binding partners (e.g. enigma for SH2B2 but not SH2B1 or SH2B3 (76); c-cbl for SH2B2, SH2B3 but only the SH2B1α isoform of SH2B1 (64, 65, 252)). The ability to cycle between nucleus and cytoplasm is a property of SH2B1 not shared by SH2B2, and represents another distinction between the two family members.

Directional transport between the nucleus and the cytoplasm occurs in an energy-dependent manner through the nuclear pore complex. For the vast majority of proteins, crossing the nuclear pore complex from the cytoplasm requires interaction with importinα (Impα) of the Impα/β complex. The nuclear localization signal endows a protein with a binding motif that is recognized by Impα (253). The main types of known NLSs are monopartite and bipartite sequences. The monopartite NLS contains a single cluster of basic amino acids

that is often preceded by a proline and followed by a hydrophobic residue, as is found in the first NLS identified within simian virus 40 Large-T antigen (PKKKRKVE) (249) (250). The bipartite NLS contains two clusters of basic amino acids separated by a variable length spacer (10-12 a.a.) (254). Although we have yet to test for the interaction, the sequence responsible for nuclear import of SH2B1β closely resembles that of a classical monopartite NLS (KPKLKKRF). SH2B1β is thus presumed to be transported into the nucleus via the Impα/β complex. Because there is substantial mixing of cytoplasmic and nuclear proteins after nuclear envelope breakdown during mitosis (253), the presence of a NES alone within a protein is not sufficient evidence to ascribe a nuclear role for that protein. In fact, an NES in the absence of an NLS is thought to function to assure that a protein stays out of the nucleus. Thus, the identification of the NLS within SH2B1, in addition to the previously described NES, adds credibility to the hypothesis that SH2B1 possesses a nuclear function.

Although SH2B1β has the capacity to constantly move between the nucleus and cytoplasm, it is for the most part detected at the membrane and within the cytoplasm. Evidence from our previous (79) and current findings suggests that the NES and NLS within SH2B1β account for its ability to undergo nucleocytoplasmic shuttling and that this shuttling is required for NGF-dependent neurite outgrowth. Mutations directed at the NES through internal deletion [GFP-SH2B1β(Δ198-268)] or point mutation [GFP-SH2B1β(L231A, L233A)] of the NES result in significant nuclear accumulation of SH2B1β, to the extent that cytoplasmic detection of the SH2B1β export mutant is relatively difficult. The finding that the ability of SH2B1β to enhance NGF-dependent neurite outgrowth is also lost when these mutations were made to the NES suggest that SH2B1β is either required in the cytoplasm or at the plasma membrane to enhance differentiation (79). However, expression of the nuclear import mutant SH2B1β [SH2B1β(mNLS)] also inhibits NGF-dependent neurite outgrowth in PC12 cells to the same extent as the previously established dominant negative

SH2B1β(R555E). This result suggests that NGF-mediated neurite outgrowth also requires SH2B1β to cycle through the nucleus.

Previous findings have implicated SH2B1β in both platelet-derived growth factor (PDGF) and growth hormone (GH)-induced membrane ruffling and motility in 3T3 F442A pre-adipocytes (74) (73). These findings raise the possibility that SH2B1β might mediate similar cytoskeletal changes in response to NGF. Most compelling is the observation that the N-terminal portion of SH2B1β directly or indirectly binds to the small GTPase Rac, and that this binding is required for GH-induced actin rearrangement (73). Even though it is thought that the small GTPase Cdc42 is responsible for neurite outgrowth and formation of the growth cone (255), Rac has been shown to be important for resisting growth cone collapse (Kuhn, Brown J. Neurosci 1999) and appropriately correct target innervation (256). Thus, a plausible ERK independent role for SH2B1β could be to function as an adapter protein that recruits Rac and possibly other small GTPases to activated TrkA complexes in order to facilitate appropriate changes to the cytoskeleton required for neurite outgrowth. However, overexpression of a truncation mutant of SH2B1β lacking the Rac binding domain is still able to enhance NGF-dependent neurite outgrowth (79). While this result indicates that Rac binding is dispensable for SH2B1β function in neurite outgrowth, until our most recent finding using the SH2B1β nuclear import mutant, there remained a distinct possibility that SH2B1β is required only at the membrane to direct NGF-induced morphological differentiation. Because the cytoplasm and plasma membrane constrained SH2B1β(mNLS) inhibits NGF-induced neurite outgrowth, we currently believe that even if SH2B1β has direct effects on the actin cytoskeleton in PC12 cells, the ability of SH2B1β to facilitate neurite outgrowth in response to NGF requires SH2B1β to cycle in and out of the nucleus.

This interpretation is in agreement with a recent finding that SH2B1β promotes more than localized changes to the cytoskeleton after NGF treatment. In fact, overexpression of SH2B1β was found to enhance the transcription of a

subset of NGF-responsive genes, several of which are biochemical markers of neuronal differentiation (124). Among the genes that displayed the most dramatic regulation by SH2B1β were uPAR, Mmp3, and Mmp10 (124). Interestingly, the protein products of these genes fall within the canonical pathway responsible for extracellular matrix (ECM) degradation, required during neurite outgrowth. The glycosylphosphatidylinositol (GPI)-linked protein uPAR is thought to initiate a proteolytic cascade through binding to and activating urokinase plasminogen activator (uPA) at the surface of the cell. The activated form of uPA converts zymogen plasminogen to plasmin, which then cleaves several of the matrix metalloproteinases, resulting in their activation (reviewed in (192)). In response to neurotrophic signals, neurons position these extracellular matrix (ECM) proteases to their growth cones, allowing the growth cone to penetrate the ECM en route to its intended target (257). Farias-Eisner et al. (122) demonstrated that NGF-induced transcription of uPAR, in particular, is an essential event for NGF-dependent differentiation of PC12 cells. Both antisense oligoncleotide mediated knockdown of uPAR transcript as well as functional inhibition of uPAR using an anti-uPAR antibody, blocks NGF-dependent PC12 neurite outgrowth (122). uPAR is an immediate-early gene (IEG) in neurotrophin-driven neuronal differentiation and its transcription is specifically induced by NGF, but not EGF, treatment of PC12 cells (122). Correspondingly, blocking uPAR function inhibited the transcription of NGF-induced secondary response genes, COX-1, type II sodium channel, Mmp1 (collagenase-1), and Mmp3 (stromelysin-1; transin-1) (122). The ability of uPAR to initiate a secondary wave of transcription important for differentiation is thought to arise from its capacity to activate intracellular signaling machinery, even though it lacks a cytosolic domain (258). Activation of uPAR by uPA is coupled to the cytoplasm through interactions with transmembrane proteins including integrins, G-protein coupled receptors (GPCR) and caveolin, resulting in the activation of a variety of kinases, including Lck, Src, FAK and ERK (258).

Because uPAR plays a multifaceted and critical role during NGF-dependent differentiation, we were particularly intrigued by our previous finding that SH2B1β influences the expression of uPAR in response to NGF (124). The overexpression of SH2B1β greatly enhances both the NGF-dependent increase in levels of uPAR transcript and neurite outgrowth. Conversely, overexpression of SH2B1β(R555E) inhibits both NGF-dependent transcription of uPAR and neurite outgrowth, compared to control cells. These data suggest that SH2B1β facilitates neuronal differentiation at least in part by enhancing the NGF-dependent transcription of the immediate early gene uPAR encoding uPAR and either directly or indirectly enhances transcription of the genes encoding MMP-3 and 10. In support of this hypothesis, we show here that knocking down the levels of endogenous SH2B1 blocks NGF-dependent neurite outgrowth in addition to inhibiting the NGF-dependent expression of uPAR, Mmp3, and Mmp10 (124). Because the expression of SH2B1β(mNLS) also blocks NGF-induced neurite outgrowth, we postulate that SH2B1β is required in the nucleus for the appropriate transcription of uPAR and potentially other genes required for NGF-dependent differentiation. Our finding that expression of SH2B1β(mNLS) fails to enhance transcription of uPAR mRNA after NGF stimulation supports the notion that nuclear import of SH2B1β is required for that transcription and suggests a more direct role for SH2B1β in NGF-induced transcription of uPAR. We also show that expression of the nuclear export mutant [SH2B1β(ΔNES)] also fails to enhance uPAR transcription after NGF treatment, which suggests that nucleocytoplasmic shuttling of SH2B1β is required for the enhanced transcription of critical NGF-target genes required for enhanced neurite outgrowth. The stable expression of the mutants SH2B1β(mNLS) (FIG. 4.8) or SH2B1β(ΔNES) (79) did not result in appreciable differences in the NGF-mediated phosphorylation of ERKs, when compared to the stable expression of SH2B1β(WT). Therefore, we believe the deficits observed in neurite outgrowth and transcription of NGF-target genes upon expression of these mutants are specific to the inability of SH2B1β to

enter and or exit the nucleus, and are not a consequence of SH2B1β(mNLS) or SH2B1β(mNES) compromising an NGF/TrkA signaling cascade. The precise mechanism by which nuclear SH2B1β facilitates the NGF-dependent induction of specific genes is unknown. However, in a previous study, overexpression of SH2B1β(WT) enhanced the nuclear translocation of FoxO1 to the cytoplasm in response to NGF (245), which raises the possibility that SH2B1β could shuttle activators or repressors of transcription into or out of the nucleus. Alternatively, one could envision SH2B1β serving as a co-activator protein which functions to recruit specific transcriptional regulators to the promoter of NGF immediate-early genes. Further study is required to determine the precise nuclear function of SH2B1β.

The finding that SH2B1β is required to shuttle into and out of the nucleus to facilitate NGF-dependent differentiation is especially interesting when considering the "dominant negative" nature of the SH2B1β(R555E) mutant. SH2B1β(R555E) was previously hypothesized to block neurite outgrowth by competing with endogenous SH2B1β for downstream effector(s) and sequestering these putative effector(s) in an inactive state (68). The current finding that SH2B1β(R555E) is unable to translocate to the nucleus and thus undergo nucleocytoplasmic shuttling, offers an additional or alternative explanation as to why SH2B1β(R555E) inhibits NGF-dependent neurite outgrowth. Nucleus excluded SH2B1β mutants would still be expected to dimerize with endogenous SH2B1β in the cytoplasm through the N-terminal phenylalanine-zipper (259), potentially preventing endogenous SH2B1β from entering the nucleus.

For proteins that contain both an NLS and NES, one would expect the balance between nuclear export and import to be subjected to some type of regulatory mechanism(s). Phosphorylation in particular has been shown to alter NLS and/or NES activity (260) (261). In SH2B1, the NLS appears more likely than the NES to be a target for regulatory post-translational phosphorylation as

the NLS is surrounded by several putative Ser/Thr phosphorylation sites. If NGF-dependent phosphorylation of the NLS proximate sites is required for the nuclear translocation of SH2B1, it could explain why the phosphorylation deficient SH2B1β(R555E) is excluded from the nucleus. Charge disruption at the critical arginine residue within the FLVR motif abolishes the phosphotyrosine binding ability of an SH2 domain (262). The arginine to glutamate mutation, therefore, abolishes the NGF-dependent association between SH2B1β(R555E) and activated and tyrosyl phosphorylated TrkA (33). As a result, the SH2B1β(R555E) is devoid of any detectable tyrosyl phosphorylation and its Ser/Thr phosphorylation is also dramatically reduced (33). The R555E mutation in SH2B1β might also alter its folding and/or binding to a separate, currently unknown protein, which results in the masking of the NLS.

The continuous compartmental cycling of SH2B1β is consistent with a newfound trend for signaling molecules. In contrast to conventional thought that signaling molecules only translocate to the nucleus after their "activation" by a particular signal, the latent unphosphorylated and "inactive" STAT2 is now thought to undergo constant shuttling between the cytoplasm and nucleus (263). Similarly, what was once believed to be a TGF-β-mediated translocation from the cytoplasm to the nucleus of the receptor-regulated Smad (R-Smad) family of proteins, has recently been shown to be a continuous cycling process independent of TGF-β stimulation (264) (265). A TGF-β-dependent phosphorylation of the R-Smads allows a nuclear R-Smad/Smad4 complex to persist, giving rise to a prolonged and observable nuclear presence (264). Conversely, functional NESs have been described for several transcription factors originally thought to localize only in the nucleus. For transcriptional regulators Oct-6, Sox9, and Sox10, disabling their nuclear export reduced their transactivation abilities (266) (267) (268). It is not clear as to what, access to the cytoplasm and nucleus affords these transcription factors.

In summary, we have demonstrated that the adaptor protein SH2B1β is required for NGF-mediated differentiation of PC12 cells in a manner distinct from

that of facilitating the activation ERKs 1 and 2. In addition to the previously described nuclear export sequence, we identified a functional nuclear localization sequence in SH2B1. Blocking SH2B1β nuclear localization resulted in the inhibition of NGF-dependent neurite outgrowth. Furthermore, the recently identified ability of SH2B1β to enhance transcription of the NGF-responsive genes uPAR, Mmp3, and Mmp10 was blocked by disrupting either the nuclear import or export of SH2B1β. These findings suggest that SH2B1β requires access to both the nucleus and cytoplasm in order to facilitate NGF-mediated morphological and biochemical changes, raising the possibility that SH2B1β directly facilitates transcription of genes required for differentiation.

Our findings are consistent with an increasing amount of recent experimental evidence that signaling molecules actively undergo dynamic translocations and reversible bindings in order for specific and successful signal transmission (269). Such findings exemplify a major departure from the classical "hardwired signaling concept" wherein receptors and their corresponding signaling/adapter proteins stay more-or-less in place while secondary messengers are actively translocated to the nucleus.

Acknowledgements

We thank Dr. Liangyou Rui for his generous gift of the SH2B1 antibody. We thank the Michigan Diabetes Research and Training Center Hybridoma Core for the αmyc antibody. We appreciate the immunoblotting advice of Dr. Lawrence S. Argetsinger, Dr. Hui Jin, and Nathan J. Lanning. We thank Barbara Hawkins for her help with this manuscript.

This Chapter has been submitted for publication in **Molecular Endocrinology** (2008) under the title "Nucleocytoplasmic Shuttling of the Adapter Protein SH2B1β (SH2-Bβ) is Required for Nerve Growth Factor (NGF)-dependent Neurite Outgrowth and Enhancement of Expression of a Subset of NGF-responsive Genes" by Travis J. Maures, Linyi Chen, and Christin Carter-Su. Linyi Chen provided FIG 4.9. I provided FIGs 4.1-4.8.

Chapter 5

SH2B1 Associates with Transcription Factor CDCA7 upon NGF Stimulation and Rescues Inhibition of Neurite Outgrowth Caused by CDCA7 Overexpression

Abstract

For several years, SH2B1β has been known to cycle in and out of the nucleus, although its precise function within the nucleus was unknown. In this report, we describe the identification of a novel nuclear binding partner for SH2B1. Using a biochemical screen in NGF-treated PC12 cells, we pulled down the putative oncogenic transcription factor CDCA7 with a TAP-SH2B1β fusion protein. The association between CDCA7 and SH2B1 was confirmed by co-immunoprecipitation of overexpressed CDCA7 with endogenous SH2B1 after NGF stimulation. The co-immunoprecipitated SH2B1 has a molecular weight of 110,000, appropriate for a highly phosphorylated form of SH2B1β or possibly a different SH2B1 isoform. The delta isoform of SH2B1 was tested for its capacity to bind CDCA7, because SH2B1δ has a similar molecular weight as the co-precipitating SH2B1. We observed steady-state localization of SH2B1δ at the plasma membrane, nucleoplasm, and nucleolus. Overexpressed CDCA7 was able to co-immunoprecipitate SH2B1δ in an NGF-dependent manner. Interestingly, we found that overexpression of CDCA7 inhibits NGF-induced neurite outgrowth and that SH2B1β is able to rescue that inhibition. In contrast, overexpression of SH2B1δ with CDCA7 caused a new NGF-dependent phenotype of large flattened cells and increased filapodia. Collectively, these

findings suggest that SH2B1β may relieve the actions of a pro-mitogenic transcription factor, allowing the cells to undergo neurogenic changes in response to NGF.

Introduction

A substantial portion of our understanding concerning the differentiation and survival of sympathetic neurons in response to nerve growth factor (NGF) has come from work using the PC12 cell line. This cell line was derived from a rat pheochromocytoma and has been used extensively as a model of both neuronal function and neuronal differentiation. Upon NGF-mediated activation of the PC12 cell's endogenous receptor tyrosine kinase TrkA, the PC12 cells undergo a dramatic transformation from a rounded appearance into a neurite bearing sympathetic-like neuron (16). Additionally, the NGF-mediated differentiation of the PC12 cells leads to the transcription of many neuronal specific markers (17) as well as a dependence upon NGF for survival, both of which have been well documented in sympathetic and sensory neurons (180). At a molecular level, several pathways relevant to the process of NGF-mediated differentiation and survival have been elucidated. In response to the NGF-induced activation and autophosphorylation of TrkA, several signaling proteins directly associate with TrkA at conserved phosphotyrosine residues (28), including phosphatidylinositol 3-kinase (PI3K), Shc (102), phospholipase Cγ (PLCγ) (104), FRS2 (243), Grb2 (270), Csk homologous kinase (CHK) (271), and the atypical PKC-interacting protein (129). Several of these proteins form signaling complexes that precisely regulate the activity of downstream targets such as ERK. Indeed, prolonged ERK signaling has been reported to play an essential role in NGF-induced differentiation as opposed to the transient activation of ERK by epidermal growth factor (EGF), which leads to cell proliferation (24-27). Active ERKs translocate to the nucleus to phosphorylate and transcriptionally activate several transcription factors, including Elk1 (272, 273) and cAMP response element-binding protein (CREB) (274). Interestingly, within an hour of NGF treatment, PC12 cells induce transcription of early growth response-1 (Egr1) (122) a known immediate-early gene of both NGF and EGF

(201, 275-277). However, within the same time period, NGF also induces transcription of the immediate-early gene VGF and urokinase plasminogen activator receptor (uPAR), neither of which are induced by EGF (122). Several subsequent studies have focused on uPAR because it is one of the only known immediate-early genes that is required for NGF-mediated differentiation. Recent characterization of the uPAR promoter revealed that NGF-induction of uPAR depends upon an AP-1 site that is preferentially bound by transcription factors JunB and c-Fos (242) whose expression is known to be induced by ER1/2 phosphorylation of . However, it is unclear how the cell translates the temporal differences in ERK activity into the resulting ligand-specific transcription causal for neuronal differentiation. For this reason it seems likely that there is an additional, as of yet unidentified, receptor specific control that works in concert with the NGF-dependent prolonged ERK activation.

We recently reported that SH2B1β, an adaptor molecule that binds to NGF-activated TrkA (33, 34), contains both a nuclear localization sequence (NLS) and nuclear export sequence (NES) and undergoes nucleocytoplasmic shuttling (79) [Maures, T., Chen, L., and CarterSu, C., submitted manuscript]. This was a surprising finding because SH2B1β was thought to localize and function only at the plasma membrane. Further examination of SH2B1β revealed that nuclear shuttling of SH2B1β is required for NGF-dependent differentiation of PC12 cells, suggesting that NGF-induced differentiation involves direct enhancement of NGF-induced gene expression by SH2B1. In support of this, the overexpression of SH2B1β was found to enhance a subset of NGF-responsive genes including the immediate-early gene uPAR. Disruption of either the nuclear import or nuclear export of SH2B1β blocks its ability to enhance uPAR transcription in response to NGF [Maures, T., Chen, L., and CarterSu, C., submitted manuscript]. We hypothesized that SH2B1β cycling between the nucleus and plasma membrane provides needed inter-compartmental communication to preferentially activate NGF-induced immediate-early genes.

To gain insight into SH2B1β's role in the nucleus, we sought to identify nuclear SH2B1 binding partners. Here we report the discovery of cell division cycle associated (CDCA7/JP01) protein as a novel nuclear binding partner for SH2B1. CDCA7 was initially identified as a c-Myc responsive gene that participates in neoplastic transformation (125). Recently, CDCA7 was found to contain a potent transactivation domain within its C-terminal end, and was concluded to function as a transcriptional regulator in the oncogenic pathway (278). CDCA7 overexpression was also reported to drive anchorage independent growth in Rat1a cells (125). While there have been no published roles for CDCA7 in neurons, several NCBI GEO microarray profiles have revealed CDCA7 transcript in mouse astrocytes as well as in PC12 cells. Interestingly, a particular GEO profile (GDS1038) demonstrated a significant reduction in CDCA7 transcript levels after forskolin treatment of PC12 cells, which is known to facilitate neurite outgrowth (279).

Consistent with this finding, we demonstrate that CDCA7 expression in PC12 cells inhibits NGF-dependent neurite outgrowth. We show that in response to NGF, CDCA7 associates with an endogenous form of SH2B1 that has a molecular weight of 110,000. This 110 kDa form of SH2B1 most likely represents a highly phosphorylated SH2B1β (the predominant isoform in PC12 cells) but could also be a larger molecular weight isoform of SH2B1 (i.e. SH2B1δ). We show that SH2B1δ is also able to associate with CDCA7 in a NGF-dependent manner. Curiously, we found that SH2B1δ localizes to the nucleolus as well as the plasma membrane. The sub-nuclear localization of SH2B1δ is most likely mediated by an isoform specific bipartite NLS in its C-terminus. We found that SH2B1β is able to rescue CDCA7 inhibition of NGF-neurite outgrowth. While SH2B1δ does not similarly restore neurite outgrowth to CDCA7 expressing cells, SH2B1δ does cause an NGF-dependent morphological change characterized by large, flattened cellular bodies and increased filapodia. Collectively, these findings suggest that SH2B1 may relieve the actions of a pro-mitogenic transcription factor, allowing the cells to undergo neurogenic changes in response to NGF.

Materials and Methods

Antibodies and reagents. Polyclonal antibody to rat SH2B1 (αSH2B1), kind gift of Dr. Liangyou Rui (University of Michigan), was raised against an SH2B1β glutathione S-transferase fusion protein and used at a dilution of 1:1000 for Western blotting (Duan, 2004). Antibodies that recognize the following proteins were used for Western blotting: IRDye800 conjugated, affinity purified anti-GFP was used at a dilution of 1:20,000 to visualize GFP (Rockland Immunochemicals Inc., Gilbertsville, PA). Ascites containing anti-mouse myc monoclonal antibody, produced by the University of Michigan Michigan Diabetes Research and Training Center Hybridoma Core, was used for immunoblotting (at a dilution of 1:10000) and immunostaining (at a concentration of 1:1000). The secondary antibodies IRDye 800 and IRDye 700 conjugated affinity purified anti-mouse IgG and anti-rabbit IgG (Rockland) and Alexa Fluor 680-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) were used at a final dilution of 1:20,000. For immuocytochemistry, anti-myc staining was visualized using Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen). NGF and rat-tail collagen I were purchased from BD Bioscience (San Diego, CA).

Plasmids. All SH2B1β constructs were subcloned into pEGFP C1 (CLONTECH Laboratories, Inc.). cDNAs encoding GFP tagged SH2B1β, GFP-SH2B1β(ΔNES) (lacking region required for nuclear export), GFP-SH2B1β(mNLS1) (mutated nuclear localization sequence), and SH2B1β(R555E) have been described previously (33) (79) [Maures, T., Chen, L., and Carter-Su,

C., submitted manuscript]. The full coding region of CDCA7 was then PCR amplified to include the flanking restriction sites, XbaI and HindIII. Using these same restriction sites, CDCA7 was subcloned into Prk5-myc vector. This same method was used to clone CDCA7 into pEGFP-C1, using BglII and HindIII as the restriction sites. The pmCherry-C1 construct was purchased from Clontech, Mountain View, CA. cDNA encoding the full length sequence of SH2B1δ (PSM/SH2-Bδ) was provided by Dr. Heimo Riedel (West Virginia University). The full coding region of SH2B1δ was PCR amplified to include the flanking restriction sites, XbaI and HindIII. The product was subcloned into the pmCherry-C1 vector using the same restriction sites. Site directed mutagenesis was performed using the primers described previously to generate mCherry-SH2B1δ(R555E) (33), and mCherry-SH2B1δ(mNLS1) [Maures, T., L. Chen, and C. Carter-Su, submitted manuscript].

N-terminal TAP-tag Construction. Rat SH2B1β was subcloned into the pEBB-NTAP vector (gift from Dr. Colin Duckett, University of Michigan). The NTAP-SH2B1β fusion protein was then digested from the pEBB vector and subcloned into the retroviral pLNCX2 vector using the restriction sites XhoI and NotI. cDNA of an EST containing the full coding region of CDCA7 was purchased from Open Biosystems (AI: BC098690.1).

Cell culture and Cell Lines. The parental PC12 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). PC12 cells were plated on collagen-coated plates (0.1 mg/ml rat tail collagen in 0.02 N acetic acid) and grown at 37°C in 10% CO_2 in normal growth medium containing Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen), 10% heat-inactivated horse serum (ICN) and 5% fetal bovine serum (Invitrogen), 1 mM L-glutamine, and 1 mM antibiotic-antimycotic (Invitrogen). PC12 cells were transfected using a Gene Pulser Xcell electroporator (400V, 500μF) in a 0.4 cm cuvette (Bio-Rad). After

5h, cells were washed with phosphate buffered saline and fresh growth medium was added.

PC12 cells stably expressing the TAP-SH2B1β fusion protein were made in the following manner: PC12 cells were subjected to retroviral infection of pLNCX2-NTAP-SH2B1β, according to Erickson et al. (280). In brief, the pLNCX2-NTAP-SH2B1β construct along with the viral packaging vectors SV-E-MLV-env and SVΨ-E-MLV (281) was transfected into 293T cells by calcium phosphate precipitation. Virus-containing medium was collected 16h after transfection and passed through a 0.45-μm syringe filter. Polybrene was added to a final concentration of 8 μg/ml. This medium was then applied to subconfluent (40%) PC12 cells. The infection protocol was repeated 3X at intervals of 12 h. When cells achieved 80% confluence, they were trypsinized, replated, and stably selected in medium containing 5 mg/ml G418 for 4 weeks. The surviving cells were cultured in normal growth medium supplemented with 5 mg/ml G418.

Differentiation of PC12 cells. To assess differentiation, PC12 cells were plated on six-well, collagen-coated plates. The cells were grown in differentiation medium (DMEM containing 2% horse serum, 1% fetal bovine serum, and 50ng or 100ng of NGF per ml medim was replaced every 2 days until cellular differentiation was assessed at day 4. Cells with neurite length at least twice the diameter of the cell body were scored as differentiated cells. The percentage of differentiated cells was determined by dividing the number of morphologically differentiated cells by the total number of cells counted.

TAP screening. PC12 cells stably expressing pLNCX2-NTAP-SH2B1β were seeded in 22 plates (15 cm) and grown to near confluence. The TAP procedure was performed as described previously (282, 283) with the following changes: cells were incubated in serum-free medium overnight and then treated

with 100 ng/ml NGF for 1 h; Cell lysis was accompanied with two rounds of flash freezing with liquid nitrogen. Proteins in the final eluate from the purification was precipitated by adding 10% TCA in acetone and incubating overnight at -20 °C. The precipitate was collected by centrifugation at 4 °C (10,000 x *g* for 30 min) and rinsed in 100% acetone. The precipitate was then submitted to the Michigan State University Proteomics Core for tryptic digestion, HPLC separation and tandem mass spectrometry (MS/MS) to determine peptide sequences.

Immunolocalization. After transfection of cDNA by electroporation the cells were incubated for 24h before cells were washed in PBS and fixed with 4% paraformaldehyde. Coverslips were mounted onto slides with Prolong (Molecular Probes). Cells were incubated with 2 ng of DAPI per ml for 10 min to visualize nuclei. The subcellular distribution of the various GFP-SH2B1 or mCherry-SH2B1 proteins was determined by fluorescence microscopy. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200) with either 20X or 40X objectives. Images were captured using a CoolSnap HQ digital camera (Roper Scientific) and viewed using MetaVue imaging software. In FIG 5.4B and C, analysis of SH2B1β and SH2B1δ was performed with an Olympus FluoView 500 laser scanning confocal microscope using a 60X oil-immersion objective and FluoView software.

Cell lysis, immunoprecipitation and immunoblotting. PC12 cells were washed three times with chilled PBSV (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na3VO4, pH 7.4) and solubilized in lysis buffer (50 mM Tris, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na3VO4, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 25 mM NaF. The lysates were then sonicated 4X with a 15 sec pulse (level 4) using a Hert Systems sonicator (Misonix Inc., Farmingdale, NY). The solubilized material was centrifuged at 16,750 x g at 4oC for 10 min. For

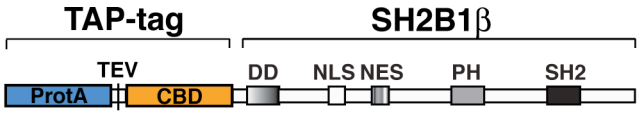
immunoprecipitations, the supernatant (cell lysate) was incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on 40 ml of protein A-agarose for 1 h. The beads were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl, 10% SDS, 10% b-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, pH 6.8). The solubilized proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, immunoblotted with the indicated antibody, and detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Immunoblots were quantified using LiCor Odyssey 2.0 software and normalized for levels of lysates as appropriate.

Results

Biochemical Screen for SH2B1 Interacting Proteins

To further understand the role of SH2B1β during NGF-mediated differentiation of PC12 cells, we employed a biochemical screen to identify binding partners of SH2B1β based on the TAP method that has been previously described (282). Briefly, SH2B1β was cloned in-frame to N-terminal TAP-tag vector consisting of an N-terminal protein-A (ProtA) and a calmodulin binding domain (CBD) separated by a tobacco etch virus (TEV) cleavage site. The resulting TAP-tagged SH2B1β construct was then subcloned into the retroviral packaging vector, pLNCX2. We created a PC12 cell line that stably expresses TAP-SH2B1β (see Methods), incubated the TAP-SH2B1β cells in serum-free medium overnight, and then treated them with 100 ng/ml of NGF for 30 min. TAP-SH2B1β was purified from whole cell lysates using two sequential chromatography columns. The first column contained IgG-beads that selectively bind and retain the protein-A tag (as shown in FIG. 5.1A). The column was then incubated with tobacco etch virus (TEV) protease, which cleaves the fusion protein, thereby freeing the calmodulin binding domain (CBD)-tagged SH2B1β and associated proteins. The eluate was then added to a column containing calmodulin beads and washed. Bound proteins were eluted with calmodulin elution buffer containing 2mM EGTA. The resulting proteins were TCA-precipitated and subjected to tryptic digestion, HPLC separation, and identification by tandem mass spectrometry (MS/MS). Several peptides corresponding to SH2B1β-binding proteins were identified. As indicated in FIG. 5.1B, SH2B1 was identified in this screen. The presence of SH2B1 is mostly likely due to the overexpression of TAP-SH2B1β, but SH2B1 is known to homodimerize and thus may also represent endogenous SH2B1 pulled down in the purification. In either case, the identification of SH2B1 serves as a proof of concept for the screen. We also identified calmodulin, which is a well know

A)



B)

Protein Name	Queries matched	gi#
SH2-B	3	gi:1354855
Calmodulin	4	gi:1827918
DNA pol (zeta)	3	gi:62666148
CDCA7	3	gi:62645239

C)

Cell Division Cycle Associated-7 (CDCA7)

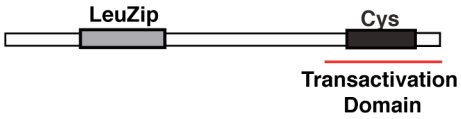


FIG. 5.1. CDCA7 was identified in a biochemical screen for SH2B1 interacting proteins.

(A) Schematic representation of the TAP-tagged SH2B1β construct. The TAP-tag consists of a Protein-A fragment (ProtA) and a Calmodulin Binding Domain (CBD), intersected by a tobacco etch virus (TEV) cleavage site. (B) The indicated proteins from the screen were trypsinized and HPLC separated before their identification by mass spectrometry. GI# of the matching peptides are indicated on the right. Query number represents the number of times the protein was identified out of 734 total queries. (C) Schematic representation of Cell Division Cycle Associated-7 (CDCA7) protein was pulled down in the screen. CDCA7 has been previously characterized as a transcription factor, containing a leucine zipper (LeuZip), a cysteine-rich region (Cys), and a C-terminal transactivation domain.

artifact of the TAP procedure. Interestingly, we identified 2 known nuclear proteins: DNA polymerase (zeta) and cell division cycle associated-7 (CDCA7) (FIG. 5.1B). In accordance with our hypothesis that SH2B1β may interact/shuttle transcriptional regulators into or out of the nucleus, we focused our efforts on CDCA7 which has been hypothesized to be a putative transcription factor.

The Identification of CDCA7 as a Novel Nuclear Binding Partner of SH2B1

CDCA7 (JP01) has only been moderately characterized in the literature. It was first identified as a c-Myc-responsive gene that is able to drive anchorage-independent growth in Rat1a cells (125). In addition to a leucine-zipper motif and a cysteine-rich region, CDCA7 was described as containing a C-terminal transactivation domain (includes the cysteine rich region) (278) (FIG. 5.1C).

To characterize CDCA7 localization and determine the morphological consequence of expressing CDCA7 within PC12 cells, we purchased an EST clone containing the full length coding sequence of CDCA7 from Open Biosystems. Rat CDCA7 was then subcloned into both myc and EGFP vectors. We transiently transfected myc-CDCA7 into PC12 cells and incubated the cells in normal growth medium (FIG. 5.2A) or serum-free medium overnight before adding 100 ng/ml NGF or EGF for 24 h (FIG. 5.2B and C). The cells were fixed and immunostained for the presence of endogenous F-actin (to show cell morphology) or myc-CDCA7. In FIG. 5.2, A, myc-CDCA7 (red nuclei) is localized exclusively in the nucleus of the transfected cell. Similarly, after 24 h of NGF stimulation, myc-CDCA7 was also nuclear. Curiously, we observed, but did not quantify at this point, the absence of observable neurites and a more rounded morphology in the cells expressing myc-CDCA7 (red nuclei) compared to the adjacent untransfected cells (FIG. 5.2, B). Moreover, in all conditions, myc-CDCA7 expressing cells often lifted from the plate to form living suspension aggregates. As expected, stimulation with EGF did not induce neurite formation

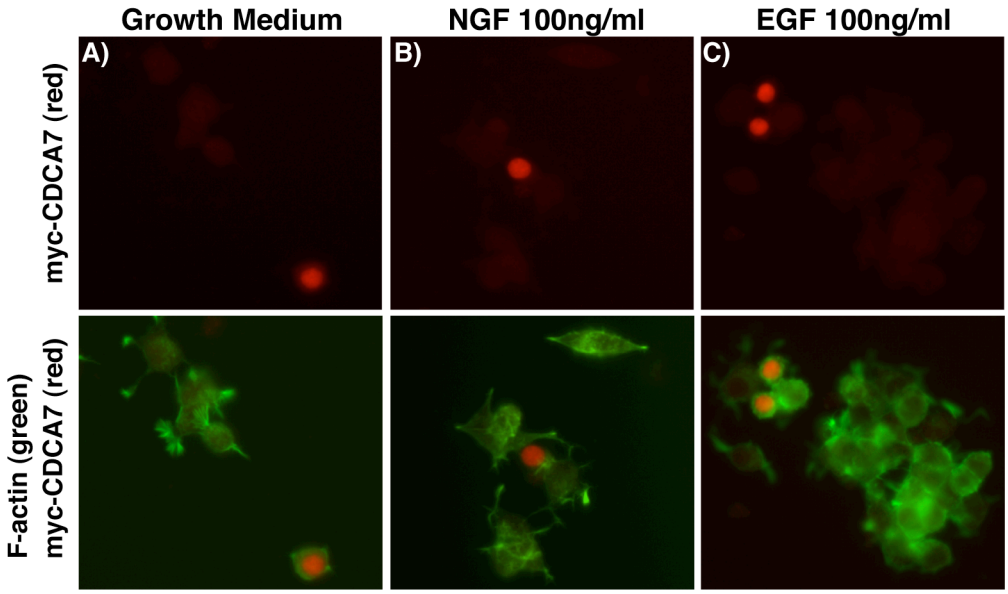


FIG. 5.2. CDCA7 is a nuclear protein in PC12 cells.
PC12 cells were transiently transfected with myc-CDCA7 (red). The cells were either incubated in growth medium for the duration of the experiment (A) or the cells were incubated in serum-free medium for 14 h before the addition of 100 ng/ml NGF (B) or 100 ng/ml EGF (C) for 24 h. The cells were fixed and stained with αmyc and anti-mouse IgG Alexa 594. All cells were also stained with Oregon Green conjugated phalloidin that recognizes F-actin to show actin cytoskeleton (green). The two images are overlayed in the bottom panels.

of the PC12 cells and myc-CDCA7 was localized to the nucleus (FIG 5.2, C). These results indicate that CDCA7 is a nuclear protein with little to no observable cytoplasmic localization. The observed decrease in adherence of the cells expressing CDCA7 appears to be consistent with its role as a transcriptional regulator that promotes anchorage independence in Rat1a cells (125). The anecdotal observation that myc-CDCA7 expression inhibited NGF-induced formation of neurites raised the possibility that CDCA7 plays an opposing role to NGF-action in PC12 cells.

Although SH2B1β has been previously described to undergo nucleocytoplasmic shuttling, the steady-state appearance of SH2B1β is within cytoplasm and at the plasma membrane (79) [Maures, T., Chen, L., and CarterSu, C., submitted manuscript]. It is currently unknown how much time SH2B1β spends within the nucleus, or how much SH2B1β is present in the nucleus at any given time. The fact that our TAP screen identified CDCA7, a *bona fide* nuclear protein, insinuated that SH2B1β is capable of forming biochemically detectable nuclear interactions. To verify the results of the TAP screen and determine whether NGF regulates the association of SH2B1 with CDCA7, we overexpressed myc-CDCA7 in PC12 cells, and incubated the cells first in serum-free medium overnight and then in the absence or presence of NGF (100 ng/ml) for 30 min. Proteins in the cell lysates were immunoprecipitated with αmyc and immunoblotted with αSH2B1. NGF stimulated the binding of myc-CDCA7 to a 110 kD form of endogenous SH2B1 (FIG. 5.3, top panel). As shown in FIG. 5.3 of lanes 1 and 2 (middle panel), in the absence of NGF, SH2B1β migrates as a ~90 kDa protein. NGF induces a significant upward shift in the mobility of SH2B1β, up to 110 kDa (FIG. 5.3, lane 3, middle panel). Previous studies indicate that the upward shift in mobility is due to phosphorylation of SH2B1β on tyrosines and serines/threonines (95). Taken together, these results suggest that myc-CDCA7 associates with a 110 kDa, highly phosphorylated, form of SH2B1β in an NGF-dependent manner.

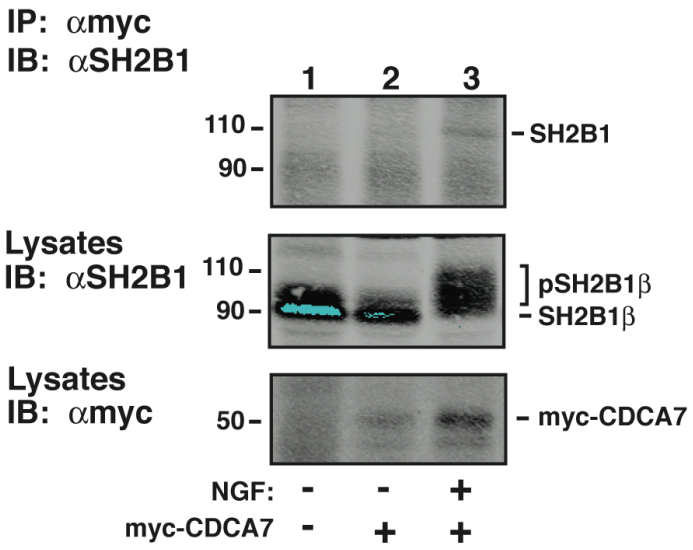


FIG. 5.3. NGF stimulates the association of CDCA7 and a 110 kDa form of SH2B1.

PC12 cells overexpressing myc-CDCA7 (lanes 2 and 3) were incubated with (+) or without (-) 100 ng/ml NGF for 30 min. Myc-CDCA7 present in cell lysates was immunoprecipitated with αmyc and coprecipitating SH2B1 was immunoblotted with αSH2B1 (top panel). present in (lanes 2 and 3)Cell lysates were immunoblotted with αSH2B1 to show expression of endogenous SH2B1, and αmyc to show levels of expression of myc-CDCA7 (bottom panel).

Identification of SH2B1δ as a Novel Nucleolar Protein in PC12 Cells

Although the aforementioned data is consistent with myc-CDCA7 associating with a highly phosphorylated form of SH2B1β, it is possible that CDCA7 associates with a different isoform of SH2B1. Of the other three SH2B1 isoforms, SH2B1α and SH2B1δ have predicted molecular weights most similar to the band identified in the co-immunoprecipitation. As previously reported, the different SH2B1 isoforms arise through alternate splicing and differ only at the C-terminal end (46). While each is predicted to have the same NLS and NES as SH2B1β, SH2B1δ possesses a unique region that contains two separate putative nuclear localization sequences (NLS)s (46). Upon further examination of the motif, we found that this region more closely resembles that of a bipartite NLS, which contains two clusters of basic residues separated by a variable length spacer (10-12 a.a.) (254). Because SH2B1δ also contains the N-terminal NLS as described for SH2B1β [Maures, T., Chen, L., and CarterSu, C., submitted manuscript], the presence of a second NLS had the potential to shift the steady state localization of SH2B1δ to the nucleus. To address this possibility, SH2B1δ was subcloned into an mCherry vector and expressed in PC12 cells. As shown in FIG. 5.4A, i, Cherry expression is distributed evenly throughout the cell. In contrast, Cherry-SH2B1δ(WT) demonstrates a striking localization to the sub-nuclear region of the nucleolus as well as the plasma membrane (FIG. 5.4A, ii). To determine which of the two NLSs was responsible for localizing SH2B1δ to the nucleoulus, we next expressed SH2B1δ carrying mutations to the SH2 domain (SH2B1δ(R555E)) and N-terminal NLS (SH2B1δ(mNLS1)) regions. Mutation of either domain halts the nuclear localization of SH2B1β, as previously described [Maures, T., Chen, L., and CarterSu, C., submitted manuscript]. In contrast to SH2B1β(R555E) and SH2B1β(mNLS1), SH2B1δ(R555E) and SH2B1δ(mNLS1) are still able to localize to the nucleus (FIG. 5.4A, iii and iv). While we have not yet directly tested it, this result indicates that the sequence resembling a bipartite NLS present in the

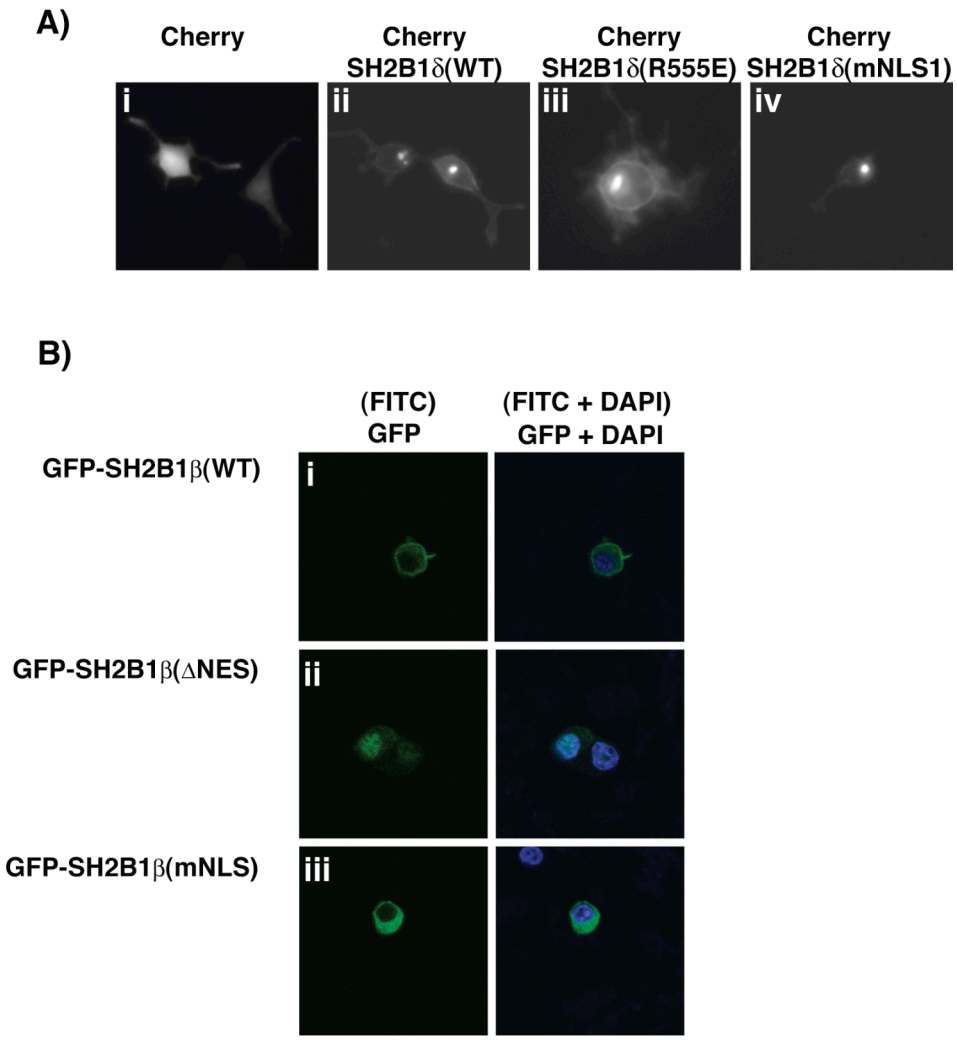


FIG. 5.4A and B. Identification of SH2B1δ as a novel nucleolar protein in PC12 cells.

(A) PC12 cells were transiently transfected with (i) Cherry vector control, (ii) Cherry-SH2B1δ(WT), (iii) Cherry-SH2B1δ(R555E), or (iv) Cherry-SH2B1δ(mNLS1). Images were taken using epifluorescence microscopy. (B) PC12 cells were transiently transfected with (i) GFP-SH2B1β(WT), (ii) GFP-SH2B1β(ΔNES), or (iii) GFP-SH2B1β(mNLS1). Fourteen h post transfection, the cells were fixed and stained with DAPI to show nuclei. GFP expression is visualized in the FITC panels on the left. The images were obtained using a laser scanning confocal microscope.

unique C-terminus of SH2B1δ most likely mediates the nuclear localization of SH2B1δ. The subcellular localization of SH2B1δ was so different from that of previously reported for SH2B1β, we reassessed the subcellular localization of GFP-tagged SH2B1β(WT), SH2B1β(ΔNES), and SH2B1β(mNLS1) using confocal microscopy. As show in FIG. 5.4B, i, GFP-SH2B1β(WT) demonstrates a steady state subcellular localization at the plasma membrane. Deletion of the nuclear export sequence within SH2B1β (SH2B1β(ΔNES)) results in its nuclear accumulation, however, SH2B1β(ΔNES) does not display any specific sub-nuclear localization to the nucleolus (FIG. 5.4B, ii). As expected, SH2B1β(mNLS1) localized to the plasma membrane an cytoplasm [Maures, T., Chen, L., and CarterSu, C., submitted manuscript].

We next assessed using confocal microscopy whether there was any positional overlap between the isoforms. In contrast to GFP-SH2B1β alone which localizes to the plasma membrane (FIG. 5.4B, i), Cherry-tagged SH2B1δ alone is found at the plasma membrane and in both the nucleoplasm and nucleolus (FIG. 5.4C, i). However, the co-expression of Cherry SH2B1δ(WT) with GFP-SH2B1β(WT), led SH2B1β(WT) to co-localize with SH2B1δ(WT) within the nucleolus in addition to the plasma membrane (FIG. 5.4C, ii). Co-expression of GFP-SH2B1β(ΔNES) with Cherry-SH2B1δ resulted in a similar nucleolar co-localization (FIG. 5.4C, iii). In contrast, GFP-SH2B1β mutants unable to undergo nuclear import (SH2B1β(R555E) and SH2B1β(mNLS)) failed to co-localize in the nucleolus with Cherry-SH2B1δ (FIG. 5.4C, 4 and 5). These data regarding SH2B1δ indicate that the SH2B1δ isoform is a novel nucleolar protein. However, SH2B1δ is also found at the plasma membrane and therefore is likely to cycle between these two locales. Our data also indicate that SH2B1β and SH2B1δ co-localize at the plasma membrane and that SH2B1β is capable of nucleolar localization in the presence of SH2B1δ.

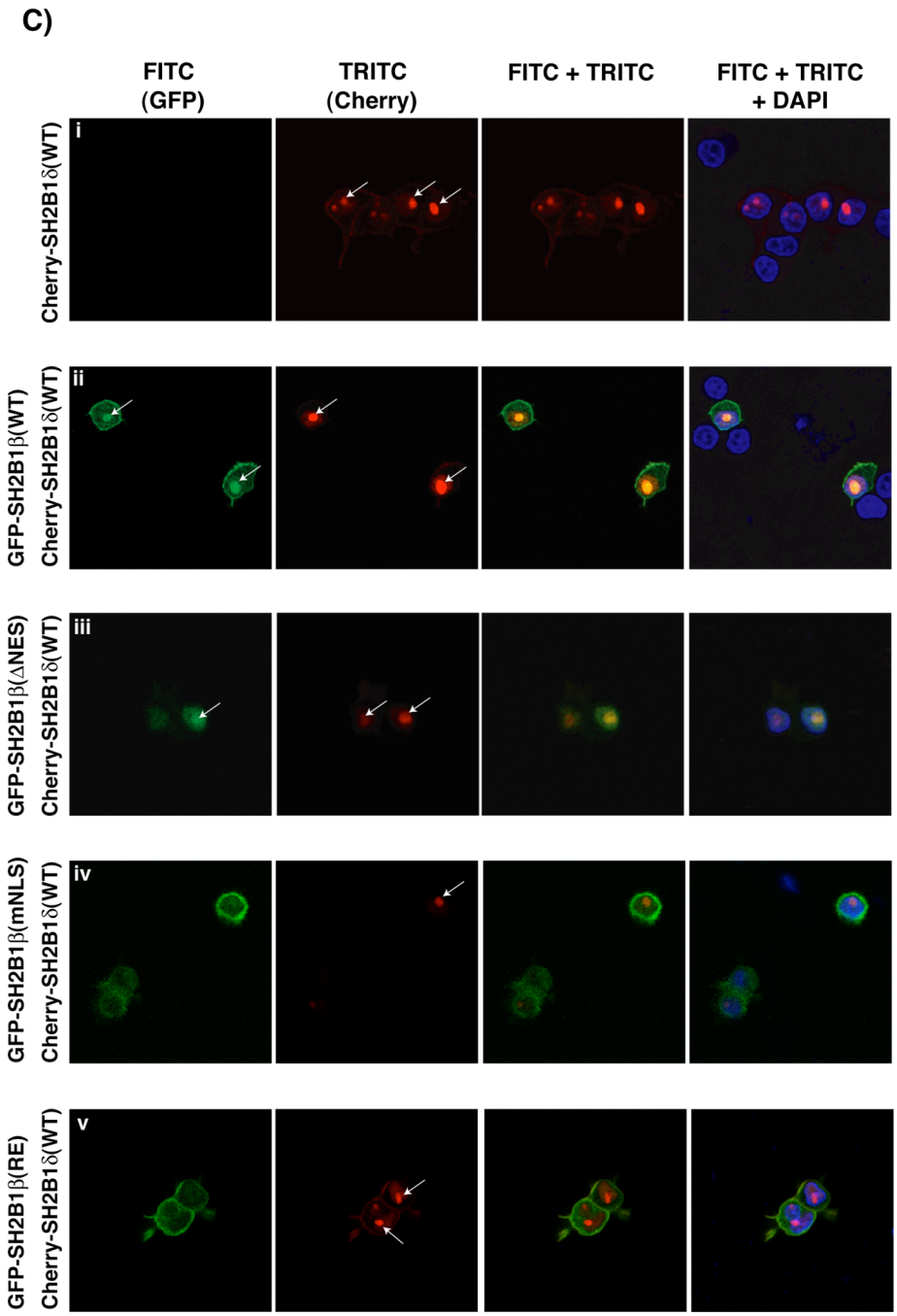


FIG. 5.4C. Identification of SH2B1δ as a novel nucleolar protein in PC12 cells.

(C) Confocal images of PC12 cells transfected with (i) Cherry-SH2B1δ(WT) alone or with the following (ii) GFP-SH2B1β(WT), (iii) GFP-SH2B1β(ΔNES), (iv) GFP-SH2B1β(mNLS1), (v) GFP-SH2B1β(R555E). As shown in Panel i), the Cherry signal (TRITC) does not cross into the GFP (FITC) channel. Co-localization of the proteins produces a yellow color in the FITC+TRITC overlay. The images were obtained using a laser scanning confocal microscope.

NGF Stimulates the Association of SH2B1δ and CDCA7

Because the δ isoform is slightly larger and demonstrates a more pronounced nuclear localization than SH2B1β, SH2B1δ was considered the most likely alternative candidate for the endogenous 110 kDa SH2B1 pulled down by CDCA7. To determine whether SH2B1δ is capable of associating with CDCA7, PC12 cells were transiently transfected with GFP-SH2B1δ and myc-CDCA7, incubated in serum-free medium for 14 h, and then in the absence or presence of NGF for 30 min. Myc-CDCA7 was immunoprecipitated from cell lysates with αmyc and the presence of co-precipitating SH2B1δ assessed by immunoblotting with αSH2B1. NGF treatment significantly enhanced the co-immunoprecipitation of GFP-SH2B1δ with myc-CDCA7 (FIG. 5.5). Importantly, αmyc did not indirectly precipitate GFP-SH2B1δ when myc-CDCA7 was not expressed (FIG. 5.5, lane 3). Interestingly, myc-CDCA7 did not precipitate the 110 kDa form of SH2B1 in addition to GFP-SH2B1δ, which migrated as a ~140 kDa. This may suggest that overexpressed GFP-SH2B1δ may out-compete endogenous SH2B1 for CDCA7 binding or the transfection efficiency of myc-CDCA7 was too low to immunoprecipitate a sufficient amount of endogenous SH2B1.

Preliminary Results Suggest SH2B1β Rescues CDCA7-mediated Inhibition of NGF-Dependent Neurite Outgrowth

As mentioned previously, experiments designed to look at the subcellular localization of CDCA7 (FIG. 5.2) suggested that overexpression of CDCA7 might inhibit the formation of neurites in response to NGF treatment. To test this more rigorously, we transiently expressed either GFP vector or GFP-CDCA7 in PC12 cells and assessed the NGF-dependent neurite outgrowth of the GFP-positive cells after 4 days (FIG. 5.6A). While ~24% of GFP expressing cells had neurites at least twice their cell body diameter, cells expressing GFP-CDCA7 had only ~7% (FIG. 5.6A). These results confirmed our earlier observation that GFP-

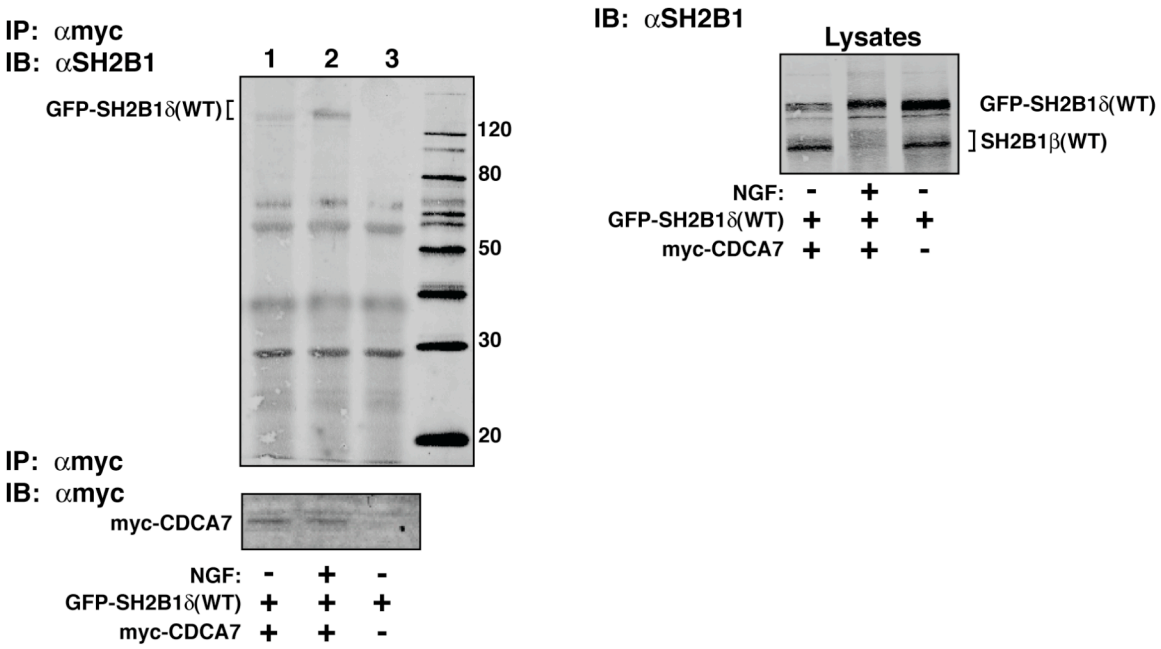


FIG. 5.5. NGF stimulates the association of SH2B1δ and CDCA7.

PC12 cells were transiently transfected with cDNAs encoding GFP-SH2B1δ(WT) (lanes 1-3) and myc-CDCA7 (lanes 1-2). The cells were incubated in serum-free medium overnight before stimulation with NGF for 30 min (lane 2). Proteins from cell lysates were immunoprecipitated with αmyc and immunoblotted with either αSH2B1 or αmyc. Cell Lysates (right panel) were immunoblotted with αSH2B1 to assess levels of GFP-SH2B1δ.

CDCA7 inhibits neurite outgrowth and suggest that CDCA7 may be a negative regulator of NGF-induced differentiation. Because the nuclear localization of SH2B1β is required for NGF-dependent differentiation, it is possible that nuclear SH2B1 is required to relieve the inhibitory action of regulatory proteins which oppose NGF effects. We therefore wanted to determine whether SH2B1β or SH2B1δ is able to rescue the inhibition of neurite outgrowth caused by CDCA7. The overexpression of myc-SH2B1β with GFP-CDCA7 not only rescued NGF-mediated neurite outgrowth but it also enhanced it to ~40% (FIG 5.5A). Co-expression of myc-SH2B1β(R555E) with GFP-CDCA7 inhibited neurite outgrowth compared to GFP alone, as expected. Next, we compared the ability of Cherry-SH2B1δ to rescue GFP-CDCA7's inhibition of NGF-dependent neurite outgrowth. After 4 days, there was no change in the morphology of the PC12 cells expressing both GFP-CDCA7 and Cherry-SH2B1δ cultured in the absence of NGF (not shown). In contrast, after 4 days of NGF exposure, there was a significant morphological change in the cells co-expressing Cherry-SH2B1δ and GFP-CDCA7. However, the phenotype that emerged from these cells was not quantifiable as differentiated neurons based on our criterion for morphological differentiation (neurite 2X length of cell body diameter) (FIG 5.6B). The cells expressing both GFP-CDCA7 and Cherry-SH2B1δ were relatively large and displayed highly active cytoskeletons. However, the processes at the leading edge of the cells were many, small, and disordered, which is more consistent with filapodia than neurite outgrowth. Typically, after 4 days of NGF treatment, the filapodia are predominately concentrated at the leading edge of the neurite's growth cone, although a discernable growth cone was not apparent in these cells (FIG 5.6). Because of the difficulty in distinguishing between neurite outgrowth and filopodia as well as the confounding problem of the large cell body size, to address whether SH2B1δ overcomes the CDCA7-mediated inhibition of NGF-induced differentiation, it will be necessary to test for the presence of neuronal

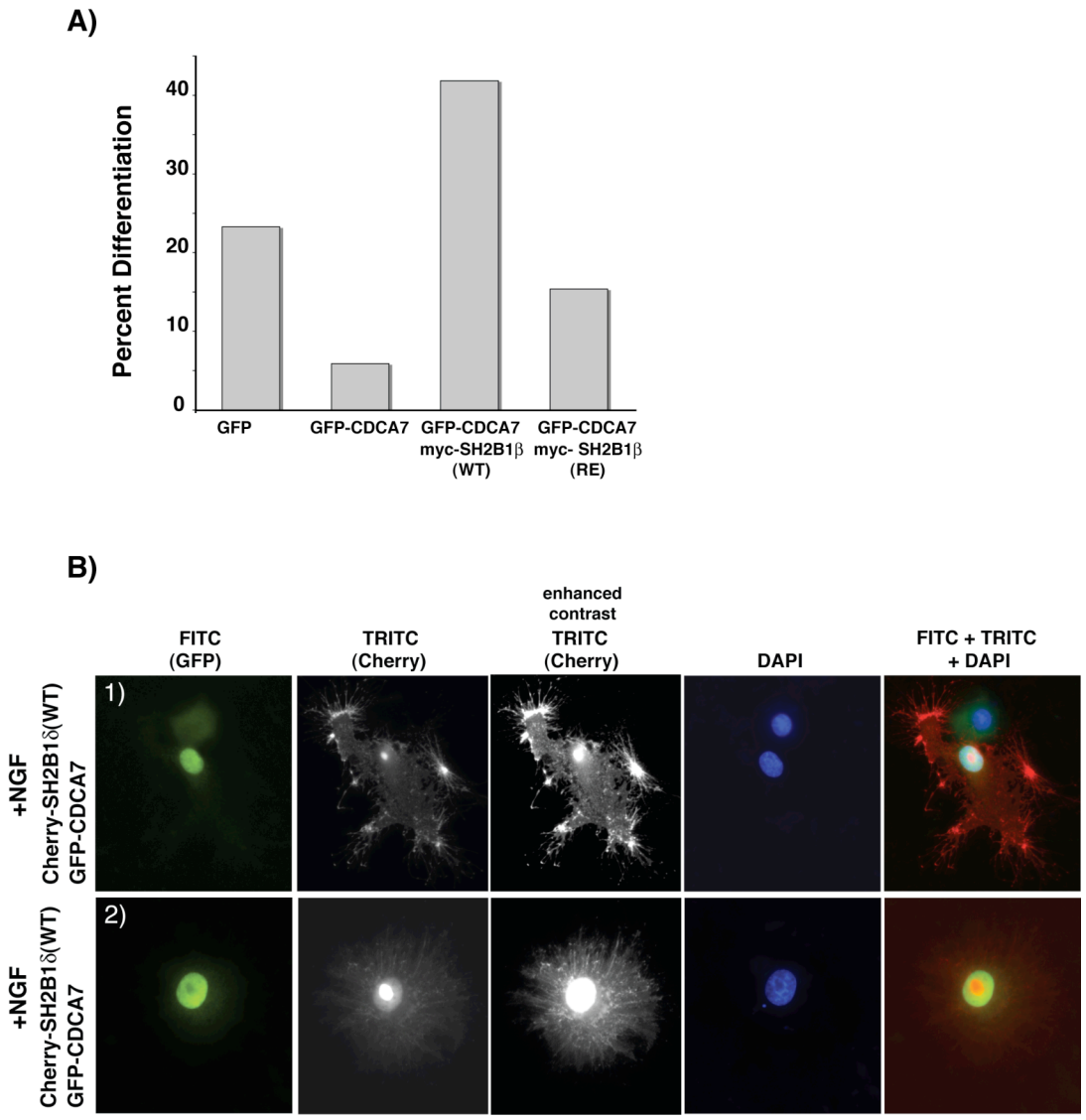


FIG. 5.6. Preliminary results suggest SH2B1β rescues CDCA7-mediated inhibition of NGF-dependent neurite outgrowth.

(A) PC12 cells transiently expressing GFP, GFP-CDCA7, GFP-CDCA7 + myc-SH2B1β, or GFP-CDCA7 + myc-SH2B1β(R555E) were incubated in serum-free medium overnight before the addition of 100 ng/ml NGF for 4 days. The percentage of differentiated cells was scored on day 4. The values represent the results from one experiment. (B) PC12 cells expressing both Cherry-SH2B1δ and GFP-CDCA7 after 4 days of NGF treatment. Panels 1 and 2 are images of representative phenotypes following the aforementioned conditions. The TRITC (Cherry) panels are shown in gray-scale for better contrast.

specific markers found only in differentiated PC12 cells. These preliminary results suggest that CDCA7's inhibition upon NGF-dependent neurite outgrowth can be overcome by the overexpresion of SH2B1β.

Discussion

SH2B1 was first identified in PC12 cells as a TrkA adaptor signaling protein that enhances NGF-mediated differentiation (33, 34). Recent findings have indicated that SH2B1β enhances and is required for NGF-dependent differentiation in a manner that is independent of and/or possibly downstream of ERK signaling (33, 79, 124) [Maures, T., Chen, L., and CarterSu, C., submitted manuscript]. The ability of SH2B1β to elicit a novel pathway independent or downstream of ERKs 1 and 2 in response to NGF is underscored by the finding that SH2B1β undergoes nucleocytoplasmic shuttling. Importantly, overexpression of nuclear import mutants of SH2B1β inhibit NGF-dependent neurite outgrowth and block the NGF-induced transcription of uPAR [Maures, T., Chen, L., and Carter-Su, C., submitted manuscript], an immediate-early gene required for NGF-mediated differentiation (122). However, without any known nuclear target for SH2B1β, the mechanism by which SH2B1β facilitates this level of regulation was only speculative.

In this report, we describe the identification of CDCA7/JPO1, a novel nuclear binding partner for SH2B1. CDCA7 was originally identified in a screen for c-Myc target genes (284). As common with other c-Myc regulated genes, CDCA7 follows a cyclic expression profile that peaks during the G1/S phase transition of the cell cycle and was thus named cell division cycle associated-7 (285). CDCA7 contains a leucine zipper motif as well as a cysteine-rich region in the C-terminus that bares resemblance to a class of DNA binding proteins in plants (286). In addition, the N-terminal region was reported to contain a transactivation domain capable of driving luciferase expression from a minimal promoter (278). While thought to be a transcription factor, CDCA7 currently has no known target genes. However, JPO2/R1, a protein with significant homology (45% identity) to CDCA7 (JPO1), demonstrates transcriptional repressor activity on the human monoamine oxidase A promoter (287).

The identification of a putative transcription factor in our screen for SH2B1β interacting proteins was particularly interesting because of an earlier finding that SH2B1β influences the subcellular localization of FoxO1 (FKHR) transcription factor (245). In the aforementioned study, SH2B1β promoted FoxO1 redistribution to the cytoplasm after NGF whereas SH2B1β(R555E), a mutant that cannot interact with TrkA, blocked NGF-dependent nuclear export of FoxO1 (245). Because SH2B1β(R555E) did not block NGF-dependent phosphorylation of FoxO1, it did not appear that the failure of FoxO1 to redistribute to the cytoplasm was a result of deficient FoxO1 phosphorylation caused by SH2B1β(R555E) (245). We recently demonstrated that SH2B1β(R555E) is unable to undergo nucleocytoplasmic shuttling [Maures, T., Chen, L., and Carter-Su, C., submitted manuscript], indicating that SH2B1β may need access to the nucleus in order to facilitate proper relocalization of FoxO1 to the cytoplasm. In general, the FoxO1 results gave rise to our hypothesis that SH2B1β may regulate gene transcription by shuttling transcription factors into or out of the nucleus.

While we have been unable to establish an interaction between FoxO1 and SH2B1β (data not shown), in this study, we have demonstrated an association between CDCA7 and SH2B1. CDCA7 associates with an endogenous form of SH2B1 that migrates as a ~110 kDa protein. Although the predominant SH2B1 isoform in PC12 cells is SH2B1β (245), the SH2B1 antibody used in this study cannot delineate between isoforms. In an unstimulated state, SH2B1β migrates at a molecular weight of ~90 kDa. A large mobility shift in SH2B1β is observed after stimulation with NGF, due to phosphorylation on Tyr and Ser/Thr residues (33, 68). Peak phosphorylation of SH2B1β is reached 15-30 min after NGF stimulation (68), resulting in the emergence of 3 or more variably phosphorylated species of SH2B1β, of which the largest runs as a 110 kDa protein. These results suggest that the 110 kDa SH2B1 co-precipitated with CDCA7 most likely represents the highest phosphorylated species of SH2B1β.

CDCA7 did not pull down any lesser phosphorylated forms of SH2B1, which indicates that the post-translational modifications of the 110 kDa SH2B1 are critical for the association. These findings raise the possibilitiy that maximal phosphorylation of SH2B1 provides a conformational change required for the association of CDCA7 with SH2B1 and/or is required for nuclear translocation of SH2B1, allowing it to come in contact with nuclear CDCA7.

One of the reasons we tested the SH2B1δ isoform for its ability to associate with CDCA7 was that it potentially contained a second NLS. SH2B1β contains a single NLS, although its presence in the nucleus is difficult to detect without inhibiting nuclear export. We hypothesized that the presence of a second NLS might shift the steady state localization of SH2B1δ into the nucleus, making it more likely to interact with nuclear CDCA7. Indeed, the overexpression of SH2B1δ in PC12 cells revealed a dramatic difference in the steady state subcellular localization of SH2B1δ versus SH2B1β..

The ability of SH2B1δ to localize within the nucleolus was, however, a surprising finding. Although first observed over 200 years ago, the nucleolus is still a somewhat mysterious organelle. The primary function of the nucleolus is thought to house ribosomal DNA (rDNA) as well as the machineries responsible for ribosomal subunit production (288). However, the nucleolar presence of SH2B1δ does not necessarily implicate SH2B1 in ribosomal biogenesis. Using confocal microscopy to analyze SH2B1δ localization within the nucleolus, we established that SH2B1δ is not homogeneously distributed (data not shown). SH2B1δ appears to localize in a similar manner as αnucleophosmin staining, which is a marker for the granular component of the nucleolus and the region containing most proteins. Several lines of evidence suggest that the nucleolus has a wide range of roles in the cell, including the regulation of mitosis, proliferation, and stress response (288).

The insulin receptor substrate-1 (IRS-1) which was thought to function only at the plasma membrane, has also been observed to localize to the

nucleolus (289, 290). In response to IGF-1, IRS-1 translocates to the nucleolus where it interacts with the upstream binding factor 1(UBF1) (289). IRS-1 stabilizes UBF-1 protein, thereby enhancing RNA polymerase-1 transcription of rRNA (290). Because cell size is often determined by the amount of available protein, an increased production of rRNA (i.e. ribosome number) gives rise to increased protein synthesis and enlargement of the cell (290). Wu et al. (290) concluded that the growth-promoting effects of IGF-1 on cell size are in part explained by the enhanced rRNA production by mediated by nucleolar localized IRS-1. Much like overexpression of SH2B1β, the expression of SH2B1δ enhances NGF-dependent differentiation, but in the absence of NGF SH2B1δ does not result in significant phenotypic changes to the PC12 cell. Only after its co-expression with CDCA7 and in the presence of NGF treatment was there observable morphological changes, without NGF, the cells displayed a typical undifferentiated morphology. The noticeably large cell size after NGF treatment may indicate that SH2B1δ and CDCA7 work in concert to stimulate rRNA synthesis.

While these findings did not elucidate a function for SH2B1δ, they did identify SH2B1δ as a novel nucleolar protein in SH2B1δ. In addition, the results from the co-expression of SH2B1δ and SH2B1β in PC12 cells may have important implications regarding what enables nucleolar localization. Unlike membrane-bound organelles, the nucleolus is a freely diffusible entity (291). However, there is considerable controversy regarding what is responsible for keeping the protein in the nucleolus. At the forefront of the debate is the questioned existence of a nucleolar localization signal (NoLS). These NoLSs usually resemble that of a bipartite NLS (292). Similarly, SH2B1δ contains a putative bipartite NLS and localizes to the nucleolus. Does SH2B1δ stay in the nucleolus because of a NoLS or because it has a binding partner in the nucleolus? SH2B1β does not contain a NoLS and it does not normally localize to the nucleolus, even after the NES is mutated (FIG. 5.4B, ii). SH2B1β only

localizes to the nucleolus when SH2B1δ is present, which is most likely a result of dimerization between isoforms (259). These results suggest two things: 1) SH2B1δ most likely localizes to the nucleolus via an NoLS; and 2) other isoforms of SH2B1 (e.g. SH2B1β) can access the nucleolus by binding to the δ isoform. This suggests that other proteins without NoLSs may reside in the nucleolus indirectly through interactions with nucleolar proteins.

CDCA7 does not appear to localize within the nucleolus. In FIG. 5.6B, 2, the FITC panel demonstrates that GFP-CDCA7 is localized throughout the nucleoplasm except for the small dark indentation in the center of the nucleus, which corresponds to the nucleolar region (FIG. 5.6B, 2 right panel). Instead, CDCA7 often appears to localize to the very outside perimeter of the nucleolus where we have identified a potential co-localization of SH2B1δ and CDCA7 (data not shown). This resembles the finding that JPO2 (closely related to CDCA7/JPO1) forms ring-like structures around what appear to be nucleoli (293). Further immunocytochemistry experiments are required in order to confidently assess the co-localization. Since only the very C-terminal portion of SH2B1 differs among isoforms, it is likely that each isoform is capable of binding to CDCA7. Thus, we were able to demonstrate an NGF-dependent association between overexpressed CDCA7 and overexpressed SH2B1δ. The pertinent question is, which of the isoforms are expressed in PC12 cells? While we have been unable to distinguish the presence of other isoforms by western blotting, RT-PCR detected transcripts of other SH2B1 isoforms (i.e. γ isoform), although to a lesser extent than SH2B1β (245).

In contrast to control PC12 cells that exhibit neurite outgrowth in response to NGF, the overexpression of CDCA7 resulted in rounded PC12 cells even after NGF treatment. Additionally, the CDCA7 cells often lifted off the plate, forming living cell aggregates in the medium. Characterized as an oncogenic transcription factor that drives anchorage independent growth, it is not surprising that CDCA7 would oppose the actions of NGF, which include cell growth, differentiation, and cessation of cell division. However, the preliminary results of

the rescue experiment suggest that overexpression of SH2B1β(WT) is able to specifically restore NGF-dependent neurite outgrowth to CDCA7 expressing cells. Moreover, SH2B1 associates with CDCA7 within 30 min of NGF and as mentioned previously, this is the critical window of time for transcriptional regulators to induce transcription of NGF-specific immediate-early genes.

Taken together, these results suggest SH2B1β shuttles continuously between the cytoplasm and nucleus of the PC12 cell. Upon NGF stimulation, TrkA and downstream kinases are activated, which give rise to a maximally phosphorylated form of SH2B1β that cycles between the nucleus and cytoplasm. However, only this maximally phosphorylated form of SH2B1β associates with CDCA7 thereby relieving the actions of a pro-mitogenic transcription factor. As hypothesized earlier for FoxO1, SH2B1β may facilitate CDCA7 inactivation by shuttling it out of the nucleus and into the cytoplasm. Although we have not demonstrated cytoplasmic CDCA7 in the presence of SH2B1, it may be rapidly degraded once in the cytoplasm as reported for p53 (294). The most obvious starting point for future experiments will be to assess whether CDCA7 affects NGF-induced transcription of uPAR, an immediate-early gene for NGF whose NGF-induced expression is dependent on SH2B1 cycling through the nucleus.

This Chapter represents a “manuscript in progress” and will be submitted for publication under the title “SH2B1 Associates with Transcription Factor CDCA7 upon NGF Stimulation and Rescues Inhibition of Neurite Outgrowth Caused by CDCA7 Overexpression” by Travis J. Maures, Linyi Chen, and Christin Carter-Su.

Chapter 6

Conclusion

The overall goal of my thesis research was to identify the mechanism by which SH2B1β facilitates NGF-dependent differentiation and survival. SH2B1 had previously been shown to associate with TrkA in an NGF-dependent manner (33, 34) and was hypothesized to enhance TrkA autophosphorylation (upon SH2B1 multimerization) (96) and recruit to TrkA signaling proteins required for activation of the Ras-dependent pathway (34). However, these proposed mechanisms could not account for the results observed using a dominant negative form of SH2B1β, lacking an intact SH2 domain (SH2B1β(R555E)). The SH2B1β(R555E) mutant, which is no longer able to bind to TrkA, inhibits NGF-mediated differentiation. However, overexpression of SH2B1β(R555E) did not appear to affect NGF-induced TrkA autophosphorylation or activation of ERKs 1 and 2 (33). These results suggested that SH2B1β(R555E) inhibits the normal function of endogenous SH2B1β at a point that is distinct or downstream from that of ERK activation by TrkA. Correspondingly, Rui et al (33). did not observe enhanced NGF-dependent TrkA or ERK1/2 activity when SH2B1β(WT) was overexpressed, although NGF-dependent differentiation was significantly enhanced. Together these results suggested that SH2B1β potentially initiates a novel pathway in response to NGF stimulation, which is required for neuronal differentiation and potentially survival.

SH2B1 Is a Positive Regulator of Nerve Growth Factor-mediated Activation of the Akt/Forkhead Pathway in PC12 Cells

The identification of SH2B1 as an important factor for NGF-mediated survival of neurons (34) led us to hypothesize that SH2B1 may facilitate PI3K signaling in response to NGF. In Chapter 2, we demonstrate that the overexpression of SH2B1β leads to a modest enhancement and prolongation of NGF-induced activation and phosphorylation of Akt. SH2B1β overexpression also enhances NGF-dependent phosphorylation of the downstream targets of Akt: GSK-3, FoxO3a (FKHRL1), and FoxO1 (FKHR) (245). The simplest explanation for this increase in Akt activity is that SH2B1β potentiates NGF-dependent TrkA activity, giving rise to increased autophosphorylation of TrkA, recruitment of PI3K to TrkA, and thus increased Akt activity. However, we do not favor such a mechanism because we have been unable to show that SH2B1β enhances TrkA autophosphorylation. There also remains the possibility that SH2B1β may directly recruit PI3K to the activated receptor or that SH2B1β inhibits dephosphorylation of Akt. In support of such a pathway, Deng et al. (66) recently demonstrated that endogenous SH2B1 interacts with the regulatory subunit of PI3K, p85, after stimulation with PDGF. Overexpression of SH2B1α enhances PDGF-activated PI3K activity and enhanced activity requires both the SH2 and PH domains of SH2B1α (66). These results suggest that SH2B1α might recruit p85 to the activated form of PDGF receptor. Although, SH2B1β is known to interact with activated forms of several receptor tyrosine kinases, the interaction between SH2B1 and p85 was restricted to PDGF receptor and was not observed with insulin or IGF-1 receptors (66). At present, it remains to be seen whether SH2B1β is able to interact with p85 after stimulation of TrkA with NGF.

If enhanced recruitment of p85 to NGF-activated TrkA/SH2B1 is required for PI3K/Akt activity, then the overexpression of SH2B1β(R555E) does not inhibit the formation of this complex since it does not inhibit NGF-dependent activation of Akt (245). This is despite the fact that overexpression of SH2B1β(R555E) is deleterious to PC12 survival (unpublished observation). Furthermore,

SH2B1β(R555E) does not affect the ability of endogenous SH2B1β to be phosphorylated by TrkA in response to NGF (68). Therefore, the mechanism by which SH2B1β(R555E) functions as a dominant negative is unlikely to be the result of its displacing endogenous SH2B1 away from TrkA. This point is valid for the ability of SH2B1β(R555E) to inhibit NGF-dependent differentiation as well as well as its ability to promote PC12 cell death. Based on these results, we felt it more likely that SH2B1β(R555E) inhibits at a point downstream of the activation of TrkA, and either downstream or independent of NGF-activation of ERKs 1 and 2 or Akt or NGF-dependent phosphorylation of GSK-3, FoxO3a, or FoxO1 by Akt.

Although, it did not appear to inhibit NGF-induced FoxO1 phosphorylation by Akt, SH2B1β(R555E) was found to affect the ability of FoxO1 to translocate from the nucleus to the cytoplasm in response to NGF after serum deprivation. Unfavorable cellular conditions, such as serum deprivation of cultured cells, lead to FoxO1 translocation to the nucleus. Upon nuclear localization FoxO1, binds to response elements of growth inhibitory and pro-apoptotic genes such as those found in the promoters of IGFBP-1 (295-298), Fas ligand (117), and Bim (299). FoxO1 nuclear localization is dynamically controlled by at least three serine/threonine (Ser/Thr), identified as Akt phosphorylation sites (117, 148, 168, 300-303). NGF has been shown to suppress FoxO1's pro-apoptotic effects through activation of Akt, which directly phosphorylates FoxO1 at these Ser/Thr (304). The FoxO1 phosphorylation by Akt leads to association with 14-3-3 protein, which results in nuclear export and possibly cytoplasmic retention of FoxO1 (117, 118, 303).

At the time of the publication of Chapter 2, we had only recently identified the capability of SH2B1β to localize to the nucleus. The finding that SH2B1β undergoes nucleocytoplasmic shuttling opened up the possibility that SH2B1β plays a direct role in the nuclear export of FoxO1, although we were unsure how SH2B1β fit into the process of FoxO1 export. Interestingly, Steve Archer in the Carter-Su laboratory identified 14-3-3 as an SH2B1 binding partner in a yeast-2-hybrid screen. Although 14-3-3 has been shown to be required for FoxO1

nuclear export (303), 14-3-3 lacks a nuclear localization sequence (NLS) and thus would require "piggybacking" by a currently unknown shuttling protein (305). Consequently, I hypothesized that SH2B1β might function to shuttle 14-3-3 into the nucleus and/or facilitate the formation of the 14-3-3/FoxO1 complex. Also, I hypothesized that SH2B1β's influence over the NGF-dependent subcellular localization of FoxO1 and/or other potential transcription factors is important for NGF-dependent differentiation.

Future Directions

My attempts to test this hypothesis, however, were met with several technical difficulties, most of which stemmed from our inability to efficiently transfect PC12 cells. In my numerous efforts to verify the association between SH2B1β and 14-3-3, I have been mostly unsuccessful in reproducing my initial finding that HA-tagged 14-3-3 co-immunoprecipitates GFP-SH2B1β in an NGF-dependent manner. The transfection efficiency of any single cDNA is approximately 20% in PC12 cells using Lipofectamine Plus reagent. Attempts to transfect two or more cDNAs dropped the transfection efficiency to under 5%. Thus, the ability to detect co-immunoprecipitation of SH2B1β and 14-3-3 was often confounded by low expression. Because the association was NGF-dependent, I was not able to use a more easily transfectable cell line such as the 293T cell line because they do not express endogenous TrkA. For these reasons, I eventually discontinued my investigation into the SH2B1β/14-3-3 interaction and focused on SH2B1β's role during neuritogenesis.

However, three advancements in our reagents/protocols have enhanced our ability to transfect and assess biochemical interactions within PC12 cells. First, I have developed a highly efficient protocol to transfect PC12 cells, using an electroporation method instead of lipid-based delivery of cDNAs (Lipofectamine). Although electroporation results in a significant amount of cellular death, I have attained transfection efficiencies of 60% and higher for two or more cDNAs. Second, in conjunction with the increased transfection efficiency, I have developed a reliable protocol to create stable PC12 cell lines. Using this method,

I have established several cell lines stably expressing various SH2B1β mutants as well as PC12 cells stably expressing an shRNA targeted against endogenous SH2B1. Third, I recently obtained a highly specific antibody for SH2B1 from the Rui laboratory. This antibody unambiguously recognizes endogenous and overexpressed forms of SH2B1 in PC12 lysates, without the need to first immunoprecipitate SH2B1.

As mentioned previously, I was also unable to detect an interaction between FoxO1 and SH2B1β. Based on the results from Chapter 2, I believe there is enough evidence to suggest that SH2B1β interacts with 14-3-3, FoxO1, or both. Therefore, utilizing these new techniques and reagents, I would like to reexamine these potential SH2B1β interactions in PC12 cells.

More importantly, I would like to more completely analyze SH2B1β's influence over FoxO1 subcellular localization. At the time of the publication of the results in Chapter 2, Dr. Chen had discovered a functional NES within SH2B1 and demonstrated that the NES was required for nuclear export of SH2B1β, but we did not know how SH2B1 localized to the nucleus in the first place. Interestingly, I found that SH2B1β(R555E) is unable to translocate to the nucleus. If nucleocytoplasmic shuttling of SH2B1β is required to facilitate nuclear export of FoxO1 after Akt phosphorylation, then this finding offers an explanation of why overexpression of SH2B1β(R555E) blocks redistribution of FoxO1 to the cytoplasm. Additionally, I have since identified the NLS within SH2B1 which is required for its translocation into the nucleus (Chapter 4). Therefore, we can now properly assess whether nuclear export of FoxO1 requires nucleocytoplasmic shuttling of SH2B1β. Flag-tagged FoxO1 would be electroporated into the PC12 cell lines stably expressing GFP, GFP-SH2B1β(WT), GFP-SH2B1β(R555E), GFP-SH2B1β(ΔNES), and GFP-SH2B1β(mNLS). The cells would then be incubated in serum-free medium overnight to induce FoxO1 nuclear localization. The cells would then be treated with or without NGF for 1 h before they were fixed, immunostained with αFlag for Flag-FoxO1, and scored for the presence of

FoxO1 in the nuclei. If NGF-dependent nuclear export of FoxO1 depends upon the nuclear presence of SH2B1β, then I would expect that the cell lines expressing GFP-SH2B1β(R555E) and GFP-SH2B1β(mNLS) would demonstrate a reduced ability to export FoxO1 from the nucleus after NGF treatment, compared to GFP control. I would, therefore, expect GFP-SH2B1β(mNLS) expressing cells to have significantly more nuclear FoxO1 after NGF treatment than GFP-SH2B1β(WT) expressing cells. If SH2B1β requires cycling between the nuclear and cytoplasmic compartments to enhance nuclear export of FoxO1, I would expect the cell lines expressing nuclear import and export mutants of SH2B1β (GFP-SH2B1β(mNLS) and GFP-SH2B1β(ΔNES) respectively) to demonstrate a similar inability to redistribute FoxO1 to the cytoplasm after NGF stimulation. However, if after treatment with NGF, the number of FoxO1 positive nuclei is the same between cells expressing GFP-SH2B1β(WT) and GFP-SH2B1β(mNLS), then I would conclude that SH2B1β is not needed in the nucleus for NGF-mediated nuclear export of FoxO1 and is not likely to be shuttling partner for FoxO1.

In conjunction with the aforementioned experiment, I would also like to address the requirement of endogenous SH2B1β for proper nuclear export of FoxO1. This experiment will also rule out the possibility that FoxO1 remains nuclear after NGF treatment due to an artifact of SH2B1β(R555E). cDNA encoding Flag-tagged FoxO1 would be electroporated into the PC12 cell line stably expressing an shRNA targeted against SH2B1 (shSH2B1) or the shRNA control cell line shControl. The cells would be incubated in serum-free medium overnight to induce FoxO1 nuclear localization. Then the cells would be treated with or without NGF for 1 h before they were fixed, immunostained with αFlag for FoxO1, and scored for the presence of FoxO1 in the nuclei. If endogenous SH2B1 is required for nuclear export of FoxO1, after NGF treatment I should observe an increased amount of FoxO1 in the nuclei of shSH2B1 cells as compared to shControl cells.

SH2B1β (SH2-Bβ) Enhances Expression of a Subset of Nerve Growth Factor-Regulated Genes Important for Neuronal Differentiation Including Genes Encoding UPAR and MMP3/10

The study presented in Chapter 3 was undertaken to identify whether SH2B1β affected NGF-induced gene transcription in PC12 cells. At the time of this study, Dr. Chen had recently established that SH2B1β contains a functional NES and persists in continual flux between the cytoplasm and the nucleus (79). The prevailing dogma at this time was that adapter proteins set up static complexes at the activated receptor, which facilitate the specific activation of the more dynamic secondary messenger that is able to physically transduce the signal from the receptor complex to the nucleus. Therefore, Dr. Chen's finding represented a departure from the dogma surrounding "cytoplasmic" adapter proteins, and a major breakthrough in our understanding of SH2B1β's function. Although the significance of SH2B1β nuclear localization was not established, the study gave rise to the possibility that SH2B1β plays a more direct role in NGF-mediated differentiation. SH2B1β lacking the region required for nuclear export was unable to enhance NGF-dependent differentiation. Dr. Chen and I hypothesized that SH2B1β directly enhances transcription of genes required for NGF-dependent differentiation.

The results of the microarray performed in Chapter 3 indicated that overexpression of SH2B1β enhances NGF-induced expression of only a specific subset of NGF-responsive genes. As expected, NGF stimulation induces transcription of hundreds of genes. If SH2B1β functioned exclusively to potentiate TrkA activity, we would expect to see a general enhancement in the transcription of the NGF-responsive genes. However, of the 511 genes whose expression doubled after treatment with NGF in control cells, only 34 demonstrated a further doubling in the SH2B1β expressing cells. Similarly, SH2B1β(R555E) expressing cells impaired by 50% or more the NGF-induced

transcription of only 153 of the 511 NGF-responsive genes. Only 6 genes showed both an enhancement of transcription by SH2B1β and impairment by SH2B1β(R555E).

Interestingly, we identified uPAR (Plaur) as one of those 6 genes whose NGF-induced expression was both significantly enhanced by overexpression of SH2B1β and impaired by overexpression of SH2B1β(R555E). We were especially intrigued by identification of uPAR, because it had previously been identified as an immediate-early gene of NGF. The transcription of immediate-early genes requires only preexisting transcriptional regulators and therefore represent the first wave of transcription required for NGF-dependent effects. Importantly, transcription of uPAR represents one of the earliest known differences between EGF and NGF-mediated gene induction and was demonstrated to be required for NGF-dependent differentiation of PC12 cells (122). In the identification of uPAR, the microarray established a potential direct target for SH2B1β. At least some of the other SH2B1β-responsive candidates identified from the microarray, such as matrix metalloproteinase-3 (Mmp3) and matrix metalloproteinase-10 (Mmp10), are most likely secondary response genes. In fact, knocking down endogenous levels of uPAR blocked the NGF-dependent transcription of several genes including Mmp3; Mmp10 was not one of the transcripts tested (122).

It is interesting to note that SH2B1β significantly affects the NGF-induced transcription of uPAR, Mmp3, and Mmp10, each of which has been previously implicated in the canonical pathway for extracellular matrix (ECM) degradation and neurite outgrowth. uPAR is thought to initiate a proteolytic cascade that includes activation of the MMPs (192). Thus, we initially envisioned that the NGF-dependent transcription of proteases enables the leading edge of growth cone to penetrate and/or move through the ECM during neurite elongation. Indeed, MMPs have been implicated in dorsal root ganglion axonal penetration through the ECM (227). These findings suggested that SH2B1β may enhance the NGF-dependent transcription of uPAR and either directly or indirectly

enhance the transcription of Mmp3 and Mmp10, thereby augmenting the proteolytic potential of the growing neurites.

However, we were perplexed by the report that uPAR protein is required for differentiation within the first two hours of NGF exposure (121). Strikingly, uPAR function can be blocked after this initial period and differentiation proceeds normally (121). While we demonstrated that uPAR is present at the plasma membrane and growth cone (FIG. 3.6B), uPAR cannot be required at the growth cone because neurites are not yet present after only two hours of NGF treatment, and we were forced to reevaluate our proposed model. The NGF-induced differentiation of PC12 cells can be divided into two distinct phases. The first phase is marked by a significant increase in transcription to provide the cell with the specialized proteins it requires for the complete differentiated phenotype. During the first phase, neurite outgrowth is a transcription-dependent process (306). After the cell generates an appropriate level of transcript, it moves into the second phase, in which elongation of the neurites no longer requires synthesis of mRNA, instead, neurite elongation requires the translation of the transcript generated in phase one (306). Essentially, uPAR appears to be required during the first phase, to "prime" the cell for differentiation, although it appears to be dispensable for the second. This would suggest that uPAR is more likely to be required for subsequent rounds of transcription in phase one, than provide a direct mechanical role for the developing neurites.

Accordingly, relatively recent publications have reported that uPAR is able to activate intracellular signaling pathways, even though uPAR is an extracellular membrane protein lacking a transmembrane and cytosolic domain (258). Activation of uPAR is tranduced to the cytoplasm through interactions with integrins, G-protein coupled receptors, caveolin (258), and PLCε (231), leading to the activation of JAK/STAT (228), Lck, Src, FAK, and the Ras/MEK/ERK pathway (258). Notably, the activation of PLCε has previously been implicated in prolonging the NGF-dependent activation of ERKs 1 and 2, and thus neurite outgrowth (307). Farias-Eisner et al. (121) favor a mechanism in which NGF-

dependent expression of uPAR is required to activate PLCε to further prolong ERK activity and facilitate subsequent rounds of transcription (121), although this has yet to be substantiated.

The results from Chapter 3 support the possibility that SH2B1β can enhance transcription of genes required for NGF-dependent differentiation. Equally as important, the results of the microarray provided us with uPAR, a potential gene target for SH2B1β.

Nucleocytoplasmic Shuttling of the Adapter Protein SH2B1β (SH2-Bβ) is Required for Nerve Growth Factor (NGF)-dependent Neurite Outgrowth and Enhancement of Expression of a Subset of NGF-responsive Genes

As previously mentioned, at the time the experiments were performed in Chapters 2 and 3, we had not yet established the region responsible for SH2B1β nuclear import. Chapter 4 describes the identification of the N-terminal NLS within all isoforms of SH2B1. Surprisingly, I also identified that a functional SH2 domain is required for SH2B1β nuclear localization in PC12 cells, as SH2B1β(R555E) is unable to translocate to the nucleus. This finding raises the possibility that the inhibition of NGF-dependent differentiation in PC12 cells overexpressing SH2B1β(R555E) is a consequence of the mutant's inability to appropriately localize to the nucleus. Taken together with the results from Chapter 3, this finding suggests that nuclear import of SH2B1β may be required for NGF-induced transcription of uPAR. Indeed, QT-PCR results described in Chapters 3 and 4 demonstrated that PC12 cells stably expressing SH2B1β(WT) greatly enhance NGF-induced transcription of uPAR, while PC12 cells stably expressing SH2B1β(R555E) significantly inhibit NGF-induced transcription of uPAR.

Importantly, a valid concern regarding the aforementioned results is whether the dominant negative effects observed in SH2B1β(R555E) expressing cells are a consequence of SH2B1β(R555E) serving as a dominant negative for

SH2B1β or for some other protein. To alleviate this concern and provide more direct evidence foa role of endogenous SH2B1 in NGF-induced neuronal differentiation, I created PC12 cells stably expressing shRNA targeted against endogenous SH2B1 as well as a control cell line. In Chapters 3 and 4, we demonstrated that the depletion of endogenous SH2B1 inhibits NGF-induced transcription of uPAR, Mmp3, and Mmp10 compared to control. Likewise, knocking down endogenous levels of SH2B1 inhibited NGF-dependent differentiation. The knockdown of SH2B1 does not alter the extent or duration of the NGF-induced phosphorylation of Akt, or ERKs 1 and 2, suggesting that endogenous SH2B1 is required for NGF-dependent neurite outgrowth in a manner that is distinct from the activation of P13K/Akt and/or Ras-ERK1/2 pathways. Clearly, the results from the SH2B1 knockdown cell line are consistent with the effects of SH2B1β(R555E) overexpression, and are consistent with the R555E mutation specifically inhibiting the function of endogenous SH2B1. Based upon my current model, I would predict that SH2B1β(R555E), unable to access the nucleus, binds to and prevents endogenous SH2B1β from entering the nucleus. In light of the aforementioned findings, I wanted to test whether nuclear localization of SH2B1β is required for NGF-induced differentiation.

To test this hypothesis, I assessed NGF-dependent neurite outgrowth of PC12 cells stably expressing a nuclear import mutant of SH2B1β lacking a functional NLS (SH2B1β(mNLS)). The results from Chapter 4 suggest that nuclear localization of SH2B1β is required for NGF-dependent neurite outgrowth. Moreover, SH2B1β(mNLS) expressing cells were also unable to enhance NGF-induced transcription of uPAR, Mmp3, or Mmp10 as compared to SH2B1β(WT). The mutation of the NLS within SH2B1β successfully blocked nuclear translocation, but did not appear to affect the NGF-dependent association with activated TrkA. SH2B1β(mNLS) seemed to be phosphorylated to a similar extent as SH2B1β(WT) in response to NGF. Also, expression of SH2B1β(mNLS) did

not alter the NGF-activated phosphorylation of TrkA or ERKs 1 and 2. Therefore, I believe the deficits in neurite outgrowth are specific to the inability of SH2B1β to enter the nucleus, and not a result of SH2B1β(mNLS) compromising an NGF/TrkA signaling cascade. Together these results clearly suggest that the ability of SH2B1β to translocate to the nucleus is important for both NGF-dependent morphological and biochemical differentiation. PC12 cells stably expressing SH2B1β(ΔNES) (lacks the nuclear export sequence) were also unable to enhance NGF-induced transcription of uPAR, Mmp3, or Mmp10 as compared to SH2B1β(WT). Collectively, these results suggest that SH2B1β requires access to both the nucleus and cytoplasm in order to facilitate NGF-mediated morphological and biochemical changes, raising the possibility that SH2B1β directly facilitates transcription of genes required for differentiation.

Because SH2B1β's transport between the nucleus and cytoplasm is an energy-dependent mechanism, it is logical to expect that this process would be subject to some level of regulatory control. The finding that SH2B1β requires a functional SH2 domain in order to translocate to the nucleus suggests that SH2B1β may first have to be phophorylated by one of the tyrosine kinases it associates with. If TrkA-dependent phosphorylation of SH2B1β was required for nuclear import, it could account for the inability of SH2B1β(R555E) to translocate to the nucleus. SH2B1β(R555E) is unable to associate with active TrkA, thus SH2B1β(R555E) is deficient in both tyrosyl and Ser/Thr phosphorylation after NGF stimulation (33, 68). However, I have been unable to observe an NGF-dependent compartmental regulation of SH2B1β. We have mutated several phosphorylation sites within SH2B1β, including all tyrosines, several predicted Ser/Thr sites, as well as Ser 96 which has previously been shown to be phosphorylated by activated ERK *in vitro* (68). None of these mutations inhibited SH2B1β nuclear accumulation in the presence of leptomycin B. However, there remain multiple Ser/Thr sites we have not yet tested and the possibility remains that multiple phosphorylation sites are required for nuclear import. However, the

pattern of continuous nucleocytoplasmic cycling by SH2B1β(WT) and nuclear exclusion by SH2B1β(R555E) is maintained whether the cells are serum deprived or treated with NGF. Therefore, if phosphorylation of SH2B1β is required for nuclear import, then these results inicated that phosphorylation of tyrosyl residue(s) within SH2B1β is nonessential and basal phosphorylation of SH2B1β is sufficient for nuclear localization.

However, it is possible that NGF-dependent phosphorylation of SH2B1β could significantly enhance the nuclear import rate of SH2B1β. Because the rate of nuclear export exceeds that of nuclear import, SH2B1β looks to be predominately located at the plasma membrane/cytoplasm. Therefore it is difficult to assess SH2B1β's rate of nuclear import without first blocking nuclear export. Interpretation of these results is complicated by the fact that the drug used to block nuclear export, Leptomycin B, is toxic and may have non-specific effects on the cells. Furthermore, Leptomycin B takes hours to be effective, thus making it difficult to assess rapid changes in rates of nuclear import or export. In the near future, I would like to attempt an experiment using fluorescence recovery after photo-bleaching (FRAP). Through conversation with Stephen Lentz, the director of the University of Michigan Morphology and Image Analysis Core, we decided that using the nuclear accumulated SH2B1β(ΔNES) may be the best chance to observe the kinetics of SH2B1β's nucleocytoplasmic shuttling. In this experiment, I would photo-bleach the nuclear signal and compare the rate of fluorescence recovery in the presence or absence of NGF.

Of course, it is also conceivable that phosphorylation does not influence nuclear import or export of SH2B1β. The subcellular localization of SH2B1β may be regulated by other post-translational modifications. It is equally possible that SH2B1β constitutively cycles between the nucleus and cytoplasm without any regulation. While this may seem inefficient, continuous cycling of SH2B1β may offer the cell a means to constantly communicate the events at the plasma membrane to the nucleus and vice versa.

That being said, I hypothesize that NGF-dependent phosphorylation of SH2B1β is required for its nuclear role. Phosphorylation of SH2B1β(WT) by TrkA at the membrane may alter its interactions with other proteins and correspondingly its function within the nucleus. Because NGF-dependent phosphorylation of SH2B1β may be required for the enhanced transcription of uPAR, Mmp3, and Mmp10, we must interpret the effects of the SH2B1β(ΔNES) mutant with caution. Based on the findings of Chen et al. (79), SH2B1β(ΔNES) is able to associate with active TrkA but at a reduced level compared to SH2B1β(WT). Thus, I believe it is unlikely that the constitutively nuclear SH2B1β mutant is phosphorylated to the same extent as SH2B1β(WT) in response to NGF. The latter is based on the finding that in contrast to SH2B1β(WT), SH2B1β(ΔNES) does not display an NGF-dependent upward shift in migration, a shift which Rui et al. (33, 68)showed was due to increased phosphorylation. Therefore, it is possible that SH2B1β(ΔNES) fails to enhance NGF-induced transcription and neurtite outgrowth because it is insufficiently phosphorylated. Presently, it remains unclear as to why SH2B1β needs access to both the cytoplasm and the nucleus. Is it because SH2B1β requires cytoplasmic phosphorylation for an active role in the nucleus, or does it require the cycling between the two compartments, possibly functioning as a shuttling protein, or is it a combination of the two? Obviously, the identification of nuclear binding partners of SH2B1β or phosphorylation site(s) within SH2B1β would help answer these questions.

SH2B1 Associates with Transcription Factor CDCA7 upon NGF Stimulation and Rescues Inhibition of Neurite Outgrowth Caused by CDCA7 Overexpression.

One of the difficulties in trying to establish a nuclear role for an adapter protein is that there are currently few analogous models to follow. While there

have been several recent reports demonstrating nuclear localization of both receptor (and receptor associated) tyrosine kinases as well as their corresponding adapter proteins, there has been little conclusive evidence concerning their nuclear function. The lack of functional data have also fueled criticisms that nuclear detection of these proteins is purely an artifact of immunostaining, cellular fractionation, and/or their overexpression. However, the example of huntingtin-interacting protein 1 (HIP1) serves as strong evidence that adapter proteins can serve to regulate transcription directly. HIP1 has a steady-state localization at the plasma membrane (308) and was initially characterized as a cytoskeletal adapter protein responsible for modulating actin dynamics (309). Interestingly, upon androgen treatment of cells, HIP1 associates with androgen receptor, translocates to the nucleus, and functions as a co-activator at androgen responsive elements of the endogenous prostate-specific antigen (PSA) promoter (308). If SH2B1β is a novel regulator of NGF-dependent gene transcription, a logical target gene is uPAR based on evidence presented in Chapters 3 and 4. There are several potential mechanisms by which SH2B1β could enhance the transcription of uPAR. In order to narrow the field of possibilities, I wanted to establish a nuclear binding partner of SH2B1β. I felt the identification of a nuclear interacting protein for SH2B1β could provide needed relevance for its nuclear function.

In Chapter 5, I discuss the identification of cell division cycle associated 7 (CDCA7/JPO1) using the tandem affinity purification method. Currently, there is only a small base of knowledge pertaining to the function of CDCA7. The CDCA7 protein was initially described as a c-Myc responsive gene that participates in neoplastic transformation (125). Intriguingly, CDCA7 was found to contain a N-terminal leucine zipper motif (286) as well as a C-terminal transactivation domain (278) and is localized diffusely throughout the nucleus (125).

Using a co-immunoprecipitation assay in PC12 cells, I was able to confirm the association between endogenous SH2B1 and myc-tagged CDCA7 in cells

treated with NGF for 30 min, but not in control cells, revealing that this association is dependent on NGF. As noted in Chapter 5, I was puzzled by the size of the endogenous SH2B1 pulled down by CDCA7 because it migrated at the high end of where SH2B1β usually migrates after NGF treatment. These results suggested to me that the band was most likely a maximally phosphorylated form of endogenous SH2B1β. However, the antibody I used cannot differentiate between SH2B1 isoforms.

Although it is unknown if SH2B1δ is endogenously expressed in PC12 cells, its molecular weight is more consistent with the observed SH2B1 band than that of SH2B1β. In addition, I had previously found that the SH2B1δ isoform was predominately nuclear in its subcellular localization. Based upon sequence analysis of SH2B1δ, I believe the increased nuclear presence of the delta isoform is due to an additional NLS within the unique C-terminal region. The presence of the C-terminal NLS had been previously speculated by Yousaf et al. (46) but had never been tested. Because all isoforms of SH2B1 contain an NES, I assumed that SH2B1δ also undergoes nucleocytoplasmic shuttling. However, the dramatic nuclear localization of SH2B1δ at steady-state would suggest there is more nuclear SH2B1δ than SH2B1β at any given time. Therefore, I thought that nuclear SH2B1δ might be more likely than SH2B1β or the other isoforms of SH2B1 that lack the second NLS to interact with nuclear CDCA7. I was able to demonstrate an NGF-dependent interaction between overexpressed GFP-SH2B1δ and CDCA7 by co-immunoprecipitation. However, I have subsequently been unable to detect endogenous expression of SH2B1δ in PC12 lysates based on the migration of endogenous SH2B1 in serum-deprived PC12 cells, which suggests that the endogenous SH2B1 pulled down with CDCA7 is most likely a highly phosphorylated form of SH2B1β, thought to be the primary isoform in PC12 cells as assessed by RT-PCR (245). However, Mr. M. Doche and I are working to establish whether SH2B1δ is present in PC12 cells by assessing whether its transcript is present.

The overexpression of SH2B1δ revealed a very interesting finding that I think is worth pursuing. In addition to the aforementioned localization to the nucleoplasm, SH2B1δ demonstrated a striking subnuclear localization to the nucleolus. It is presently unclear what function SH2B1δ provides the cell, although insulin receptor substrate-1 IRS-1 was recently shown to translocate to the nucleolus upon IGF-I stimulation (289, 290). The IGF-I-dependent localization of IRS-1 to the nucleolus is thought to enhance the production of rRNA which functions to facilitate cell growth through increased protein synthesis (290). It will be interesting to examine whether SH2B1δ similarly facilitates cell growth through increased protein synthesis in PC12 cells if they express endogenous SH2B1δ or in other cell lines that do express SH2B1δ.

It is also interesting to speculate whether FRS2, which is in the same family of adapter proteins as IRS-1, also translocates to the nucleus. The ability of IRS-1 to localize to the nucleus is thought to depend upon the nuclear translocation of a large complex of signaling molecules (290). Considering that IRS-1 is known to interact with SH2B1 (93) it seems possible that SH2B1 may be included along with IRS-1 during its nuclear translocation. This idea is highlighted by the fact that IRS-1 and SH2B1δ both demonstrate a unique ability to localize to both the plasma membrane and the nucleolus. As discussed previously, FRS2 competes for the Shc binding site on activated TrkA and has been suggested to mediate cellular differentiation versus proliferation in response in response to NGF (243). We are currently investigating the possibility that FRS2 undergoes an NGF-dependent nuclear translocation and/or co-localizes with SH2B1 in the nucleus.

Included in Chapter 5 are my preliminary data concerning the function of CDCA7 within PC12 cells. In my first experiments to test the subcellular localization of CDCA7, I discovered that overexpression of CDCA7 dramatically inhibits NGF-dependent differentiation of PC12 cells. I initially questioned the significance of this finding. CDCA7 expression is thought to peak during the G/S transition of the cell cycle, thus it has been postulated that CDCA7 is a

proliferative transcription factor (125, 278). Conversely, NGF treatment is known to move asynchronous populations of PC12 cells into a G0/G1 arrest (310). Therefore, it was not surprising to see CDCA7 oppose the action of NGF. After establishing an NGF-dependent association between SH2B1 and CDCA7, I decided to test whether SH2B1β expression could rescue the inhibition of NGF-dependent differentiation by CDCA7. While the data in FIG. 5.6B are preliminary, the results suggest that overexpression of SH2B1β is able to restore NGF-mediated neurite outgrowth to cells overexpressing CDCA7.

As noted above, after 30 min of NGF stimulation, CDCA7 interacts with a highly phosphorylated form of SH2B1, which is most likely SH2B1β. The fact that CDCA7 did not also pull down any lesser phosphorylated species of SH2B1 indicates that this interaction is dependent upon maximal phosphorylation of SH2B1. Therefore, one potential mechanism by which SH2B1 enhances transcription of NGF-responsive genes is that NGF stimulation results in the transient formation of a highly phosphorylated form of SH2B1, which is able to bind and inactivate CDCA7. This led me to the hypothesis that CDCA7 is a repressor of essential NGF immediate-early genes, such as uPAR. The NGF-dependent inactivation of CDCA7 by SH2B1 would allow for the induction of uPAR in a temporally specific manner. Intriguingly, EGF stimulation is unable to promote tyrosyl phosphorylation of SH2B1β (33), thus I would not expect SH2B1β to associate with CDCA7 in response to EGF. Whether this corresponds to the differential ability of NGF but not EGF to induce uPAR, remains to be determined (FIG. 6.1).

Collectively, the results from Chapter 5 suggest that SH2B1 associates with a nuclear protein and putative transcription factor, CDCA7, in an NGF-dependent manner.

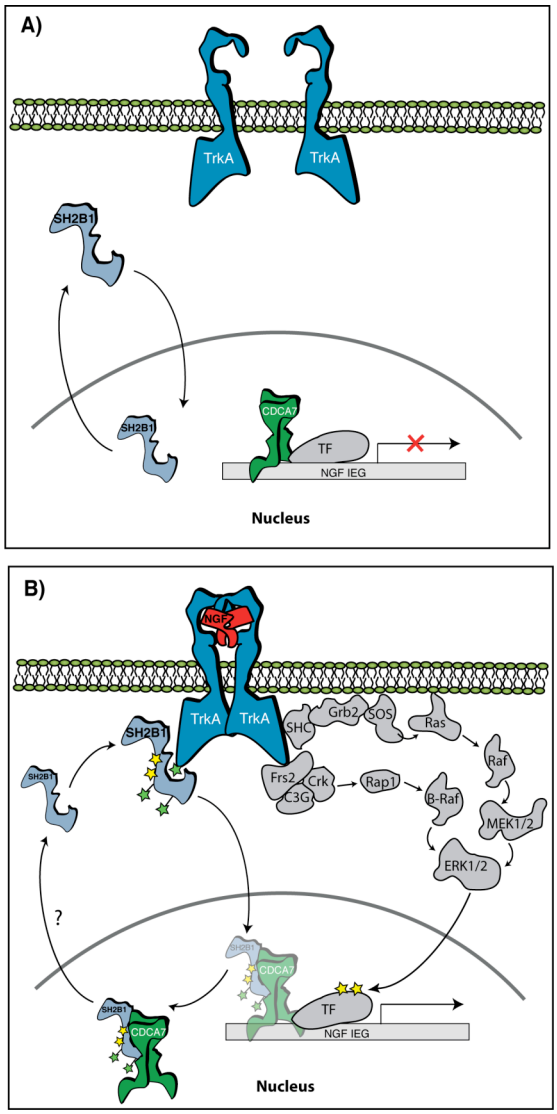


FIG. 6.1 Does SH2B1 function to inactivate CDCA7 transcriptional repression of specific NGF immediate early genes?

(A) In the absence of NGF, TrkA is inactive and SH2B1β cycles between the nucleus and cytoplasm. (B) NGF-activated TrkA stimulates the assembly of signaling pathways responsible for the prolonged activation of ERKs 1 and 2. Upon activation, ERKs 1/2 translocate to the nucleus where they phosphorylate and activate several transcription factors (TF) that are required for the induction of NGF-specific immediate early genes (NGF IEG), such as uPAR. As suggested by the results of Chapter 4, SH2B1β is required to shuttle in and out of the nucleus to enhance the transcription of NGF-induced genes required for differentiation. Our hypothesis predicts that CDCA7 functions as a transcriptional repressor of NGF-specific IEGs. NGF-dependent phosphorylation of SH2B1β (yellow and green stars) induces an association with CDCA7, which may inactivate CDCA7 repression of NGF-specific IEGs, allowing for their transcription. SH2B1β may disrupt CDCA7 binding at the promoter or may shuttle CDCA7 to the cytoplasm (?). Because EGF stimulation does not lead to SH2B1β phosphorylation, EGF would not be expected to induce the association of SH2B1β with CDCA7, thus allowing CDCA7 transcriptional repression of NGF-specific IEGs to persist.

Experimental Limitations

Unfortunately, for every experimental system there is an experimental limitation. Throughout my work, I have relied upon the overexpression of proteins. The function of a particular endogenous protein may depend upon specific stoichiometric interactions with other binding partners, which would obviously be altered upon overexpression. Therefore, overexpression can inhibit endogenous proteins and lead to functional artifacts. In order to reduce the potentially adverse effects of overexpression, we have employed the use of several stable cell lines, which exhibit a level of expression more closely aligned with that of an endogenous protein. Additionally, the development of better antibodies has enabled us to more easily and accurately identify endogenous proteins. However, there are times when analysis of the endogenous protein is not possible (i.e. no antibody or when mutants are required). In these cases, it is necessary to include all controls and leave open the possibility that the results are artifactual. We have made an effort to address these concerns when applicable. For example, in Chapters 3 and 4, we used RNAi to knockdown endogenous SH2B1. We were pleased to see that knocking down endogenous SH2B1β led to similar effects as seen in cells overexpressing the dominant negative SH2B1β(R555E).

The utilization of fluorescent tags such as EGFP and mCherry has enabled us to follow and characterize dynamic subcellular movement of SH2B1β in living cells. The obvious downside of creating such fusion proteins is that the fluorescence tag may inhibit the protein's function. The proteins encoding the fluorescent tags are relatively large (~28 kDa) and may sterically hinder proper folding of the protein of interest and/or its association with other molecules. Unfortunately, the SH2B1 antibody is not optimal for immunocytochemistry, thus SH2B1 requires tagging with a known epitope. In hindsight, I would have preferred to use the smaller myc-tag for the SH2B1β constructs to characterize their nuclear function. Indeed, if SH2B1β functions as a co-activator, the bulky GFP tag could potentially block recruitment of transcriptional regulators. In order

to combat this, I am remaking the majority of my SH2B1β constructs in myc-tagged vector. However, the myc -vector is not as amenable to the formation of stable cell lines as the GFP vector. The GFP-tag enhanced our ability to create stable lines in PC12 cells by allowing us to pool together GFP-positive cells at the flow cytometry core. I am currently analyzing the possibility of engineering a vector that would contain the GFP tag and a myc-tagged construct, separated by an internal ribosome entry site (IRES). Using this method we could still sort for GFP positive cells to make stable lines, but SH2B1β would be conjugated to the myc epitope and not GFP.

Although the PC12 cell model is a powerful system to establish molecular mechanisms of sympathetic differentiation, PC12 cells have a tumorigenic origin. One of the most significant criticisms regarding the PC12 model is that PC12 cells can undergo reversible morphological differentiation upon NGF removal (16). The lack of complete terminal differentiation of PC12 cells represents a significant difference compared to their true sympathetic neuron counterparts, which require NGF for survival (11). For this reason, we would like to assess the physiological relevance of SH2B1β's nuclear role using an animal model. This will be explored in more detail in Future Directions.

Future Directions

Identification of NGF-dependent Serine and Threonine Phosphorylation Sites Within SH2B1β.

As hypothesized in Chapters 4 and 5, the NGF-dependent phosphorylation of SH2B1β may influence its ability to translocate to the nucleus and/or facilitate the interaction with CDCA7 and other potential nuclear binding proteins. The Carter-Su lab has identified three sites of phosphorylation in SH2B1β, including Tyr 439 and Tyr494 which are phosphorylated by JAK2 (67) and Ser96 which is phosphorylated in response to NGF (68). However, mutation of these sites (68) does not reduce SH2B1β's ability to translocate into the

nucleus, nor does it affect SH2B1β enhancement of NGF-dependent neurite outgrowth (data not shown). Importantly, we have evidence to suggest that these three sites are not the only/major sites of phosphorylation in response to NGF stimulation (68) (Dr. Linyi Chen, unpublished data). Therefore it is critical that we establish the other sites of phosphorylation in SH2B1β.

To ascertain these and other unknown post-translational modifications of SH2B1β in response to NGF, I would like to purify SH2B1β from PC12 cells after 15 minutes NGF stimulation and submit it to the Michigan State University Proteomics Core or University of Michigan Peptides and Proteomics Core for analysis by mass spectrometry. Through communications with Doug Whitten from the Michigan State University Proeomics Core, I have developed a preliminary protocol that is fairly similar to the purification and mass spectrometry performed in Chapter 5. This procedure would make use of the preexisting stable line expressing TAP-tagged SH2B1β. Briefly, NGF-treated lysates from these cells would be incubated in a column of IgG conjugated beads. The column would then be subjected to several high stringency washes. Instead of digesting SH2B1β from the IgG column using TEV protease, which results in a significant loss of protein, the SH2B1β-bound IgG beads themselves would be submitted. Doug Whitten uses a protocol that can digest the protein from the beads efficiently and then analyze the resultant protein by MS/MS using a Finnegan LTQ FTMS. The University of Michigan Peptides and Proteomics Core also have a similar mass spectrometer, although for identification of post-translational modification, they often send the samples to the Michigan State University Proteomics Core. The potential problem with this method is obtaining the appropriate amount of sample required for accurate detection. However, the TAP procedure in Chapter 5 was performed using 22, 15 cm culture plates of subconfluent PC12 cells. We have the capacity to significantly increase the number of cells if the level of protein is insufficient. More importantly, the most time-consuming, technically challenging, and yield-reducing aspects of the TAP

assay (TEV cleavage and calmodulin column purification) will be eliminated from this procedure.

Assuming the successful identification of phosphorylation sites within SH2B1β, I will make corresponding single residue mutants (i.e. Tyr to Phe, and Ser/Thr to Ala). Additionally, I will make phospho-mimetic mutations for any identified Ser/Thr phosphorylation sites (i.e. Ser/Thr to Asp/Glu). These mutants will be expressed in PC12 cells and evaluated for their ability to undergo nucleocytoplasmic shuttling, enhance NGF-induced transcription of uPAR, associate with CDCA7, and enhance NGF-dependent differentiation. In doing this, I hope to establish sites of phosphorylation that are required for the nuclear translocation of SH2B1β and/or phosphorylation required for the CDCA7/SH2B1β interaction.

Does CDCA7 Specifically Inhibit NGF-dependent Differentiation of PC12 cells?

As described in Chapter 5, the overexpression of CDCA7 dramatically inhibited NGF-dependent differentiation. While CDCA7 has been described as a putative transcription factor, it presently has no known target genes. Interestingly, a closely related protein of CDCA7 (JPO1), JPO2, has been described to repress transcription of monoamine oxidase (287). Thus, one of the more attractive possible mechanisms by which CDCA7 inhibits NGF-dependent differentiation is that CDCA7 represses the transcription of uPAR or other NGF-regulated immediate-early genes. To test this hypothesis, I will create dominant-negative mutants of CDCA7 (dnCDCA7) lacking either the residues required for leucine zipper-mediated dimerization or deletions of the transactivation domain. Disrupting the hetero/homodimerization has been demonstrated to result in dominant-negative forms of c-Myc (311).

I will express a vector control, CDCA7(WT), and dnCDCA7 in PC12 cells and assess the ability to: 1) repress/activate a uPAR promoter-driven luciferase construct with and without NGF stimulation; 2) repress/activate NGF-induced

transcription of uPAR; and 3) inhibit/enhance NGF-dependent differentiation. If I find that expression of CDCA7(WT) inhibits, while dnCDCA7 enhances, NGF-induced transcription of uPAR, I could use the uPAR promoter-luciferase construct to identify the affected promoter region. There are already several transcription factor binding sites mapped out for the uPAR promoter (242).

However, it is quite possible that CDCA7 has no direct effect on uPAR expression. For this reason, I would also like to map the region(s) required for SH2B1β/CDCA7 binding. By establishing the region(s) of interaction, I could test whether the SH2B1β/CDCA7 association is required for rescuing CDCA7's inhibition of NGF-dependent neurite outgrowth. Because the interaction between CDCA7 and SH2B1 most likely depends upon successful phosphorylation and/or translocation of SH2B1, I will map the interaction by making truncation/mutations to CDCA7 only. The interactions will be analyzed using co-immunoprecipitation in PC12 cells.

Do SH2B1$^{-/-}$ Mice Demonstrate Defects in Sympathetic Nerve Innervation of Target Organs?

In our PC12 cell model, we have demonstrated that SH2B1 is required for NGF-dependent neurite outgrowth. In order to complement our molecular work, we must address whether SH2B1 is required for sympathetic neuron development using a mouse model. As mentioned in Chapter 1, SH2B1$^{-/-}$ mice are viable, although they develop obesity, type 2 diabetes, and demonstrate reduced fertility (98). The SH2B1$^{-/-}$ mice, however, were not analyzed for deficits in sympathetic neuron development. Because the mice are maintained in the highly controlled atmosphere of the laboratory, it is conceivable that the SH2B1$^{-/-}$ mice could have dramatic developmental defects within their sympathetic nervous system without an obvious phenotype.

As discussed in Chapter 1, NGF is required for both the survival as well as proximal axonal extension of sympathetic neurons (13). If SH2B1 is truly required for the NGF-dependent signaling in sympathetic neurons, I would expect

to see a reduced number of sympathetic neurons at distal organs in the SH2B1$^{-/-}$ mice. To test this hypothesis, SH2B1$^{+/+}$ and SH2B1$^{-/-}$ mice at P0 will be anesthetized and sacrificed. Whole organs, including heart, lungs, trachea, stomach, small/large intestine, liver, pancreas kidney, bladder, and gonads will be surgically removed and fixed in 4% paraformaldehyde. Tyrosine hydroxylase is the rate-limiting step in catecholamine biosynthesis (312) and is a marker for sympathetic neurons. Therefore, whole-mount diaminobenzidine (DAB)-tyrosine hydroxylase immunochemistry would be performed on organs collected from the SH2B1$^{+/+}$ and SH2B1$^{-/-}$ mice. Sympathetic innervation of the aformentioned organs will be compared between SH2B1$^{+/+}$ and SH2B1$^{-/-}$ mice as described previously (13).

Despite the requirement for SH2B1 in NGF-dependent neurite outgrowth, we may not observe a phenotype. If we do not see a significant difference between sympathetic neuron numbers within the organs of SH2B1$^{+/+}$ and SH2B1$^{-/-}$ mice, we would conclude that SH2B1 is not required for sympathetic neuron development. This result would suggest that the sympathetic neuron is able to compensate for the loss of SH2B1.

However, if the organs from the SH2B1$^{-/-}$ mice do contain a reduced number of sympathetic neurons, we would conclude that SH2B1$^{-/-}$ is required for proper sympathetic development. Since SH2B1 is predicted to play a role in neuronal survival and neurite outgrowth, this initial finding would not differentiate between neurons lost due to apoptosis at an early stage (before axonal projection) or apoptosis resulting from a defect in axonal extension and/or subsequent failure to innervate target organs. As demonstrated by Crowley et al. (11), the sympathetic neuron's dependence upon NGF for survival leads to dramatically reduced neuronal numbers within the superior cervical ganglion of NGF$^{-/-}$ mice. Therefore, it would be necessary to evaluate the initial neuronal numbers within the sympathetic chain of the SH2B1$^{-/-}$ mice. A reduced neuron count within the sympathetic chain would be suggestive that the neuroblasts are unable to properly migrate from the neural tube to form the sympathetic ganglion

or that the neuroblasts are unable generate sufficient numbers, possibly due to increased apoptosis. In contrast, if the neuronal counts at the sympathetic chain are similar between $SH2B1^{+/+}$ and $SH2B1^{-/-}$ mice but the $SH2B1^{-/-}$ mice have reduced innervation of target organs, then this would suggest that SH2B1 is important at a later stage, possibly for axonal extension and/or innervation of the sympathetic neuron. Further studies using a $Bax^{-/-}$ /$SH2B1^{-/-}$ double knockout could be used to discern whether SH2B1 is required for neuronal survival, axonal extension, or target innervation.

Summary

The overall goal of my thesis research was to identify the mechanism by which SH2B1β facilitates NGF-dependent differentiation and survival. I have provided evidence that suggests SH2B1β plays a role in the nuclear export of pro-apoptotic transcription factor FoxO1 after NGF treatment. I have identified that SH2B1 is required for NGF-dependent neuronal differentiation and NGF-induced transcription of uPAR, an essential primary response gene required for NGF-dependent neurite outgrowth. In addition, I have provided substantial evidence that SH2B1β requires cycling between the nucleus and cytoplasm to facilitate NGF-induced transcription of genes responsible for neurite outgrowth. Finally, I have provided evidence suggesting that SH2B1β interacts with a nuclear protein and putative transcription factor CDCA7 after NGF stimulation. Future studies are required to clarify the importance of this interaction. While these molecular mechanisms were elucidated in PC12 cells, an established model for sympathetic neurons, we believe the significance of SH2B1's nuclear localization may extend beyond the context of NGF. SH2B1 is an adapter protein for several receptor and non-receptor tyrosine kinases thus we would predict that the nuclear function of SH2B1 may also be relevant to the biological actions of many other ligands.

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