EFFECTS OF HUMAN OBESITY-ASSOCIATED MUTATIONS ON CELLULAR ACTIONS OF THE ADAPTER PROTEIN SH2B1

by

Michael Edgar Doche

**A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in The University of Michigan
2013**

Doctoral Committee:

**Professor Christin Carter-Su, Chair
Associate Professor Jiandie Lin
Professor Ormond MacDougald
Associate Professor Liangyou Rui
Professor Jessica Schwartz**

© Michael Edgar Doche

All rights reserved

2013

Acknowledgements

I would like thank my mentor, Christin Carter-Su. Her passion for science is only eclipsed by her passion for mentoring. Christy has taught me more about science and life in general than I could ever remember. I am truly fortunate to have Christy as a role model.

I would like to thank my committee members Jiandie Lin, Ormond MacDougald, Liangyou Rui, and Jessica Schwartz. Your advice and guidance has been well appreciated. Jessica Schwartz and Liangyou Rui have been particularly great in sharing reagents and ideas that were indispensible for my thesis work.

I would like to thank past and present members of the Carter-Su Lab. Larry Argetsinger and Joel Cline keep the lab operating and their hard work and dedication have been greatly appreciated. Grace Jin, Travis Maures and Nate Lanning were wonderful colleagues and made my transition into the Carter-Su Lab a great experience. A particular debt of gratitude goes to Barbara Hawkins for all that she has done during my graduate career. I would like to thank members of the Physiology administration staff, especially Michelle Boggs, who has kept me organized and informed.

Having a good group of friends in Ann Arbor has made being so far from home more tolerable. Matt and Kristine Sikora, Brendon and Lyndsey Ladd,

Chris and Beth LaPensee, Derek Simon, and Matt and Shelby Campbell have all been a part of my “Michigan Family” and I will cherish the bonds I have made with them. I need to give particular recognition to Matt Campbell who has provided me with housing and friendship while we finish our graduate careers.

I would like to thank my undergraduate mentor from UCLA, Dr. Osvaldo Rey and my partner while we worked in his lab Romeo Papazyan. Romeo is the person who got me interested in research and Osvaldo provided me with an opportunity to do exciting molecular biology research for the first time in my life. I would definitely not have pursued a Ph.D if it were not for them.

I would like to thank my beautiful girlfriend Kimberly Barker. Her patience, understanding, and love are boundless. I would like to thank Marc, Roxanne, Aleyna, Kyle, and the little one for reminding me what is important in life. Finally, the people who have been there through out my life and have always supported me, my parents Ed and Maryse Doche. I could not have asked for or imagined more loving and supportive parents.

Table of Contents

Acknowledgments……………………………………………………………………ii

List of Figures…………………………………………………………...………vi

List of Tables…………………………………………………………….………..x

Abstract……………………………………………………………………………xi

Chapter 1………………………………………………………………………….1

The Genetics of Obesity Study and Monogenic Causes of Obesity……….....1

The Adapter Protein SH2B1……………………………………………………..5

PC12 Cells: Cell Culture Model of Neuronal Differentiation.…………...……..15

Chapter 2…………………………………………………………………………18

Human *SH2B1* mutations are associated with maladaptive behaviors and obesity…………………………………………………………….…………..……..18

Abstract……………………………………………………….…………………...18

Introduction………………………………………………………….……………19

Results and Discussion……………………………..……………………….……..21

Methods……………………………………………………………………………39

Chapter 3…………………………………………………………………………44

Effects of human mutations on cellular actions of SH2B1 alpha……….……44

Abstract………………………………………………………………………......44

Introduction………………………………………………………………………46

Materials and Methods……48

Results……51

Discussion……66

Chapter 4……68

Effects of human mutations on cellular actions of SH2B1 delta……68

Abstract……68

Introduction……70

Materials and Methods……72

Results……75

Discussion……96

Chapter 5……100

The adapter protein SH2B1 and growth hormone-induced gene expression……100

Abstract……100

Introduction……102

Materials and Methods……106

Results……109

Discussion……117

Chapter 6……120

Conclusion……120

Summary and Future Directions……120

References……135

List of Figures

FIG. 1.1. Schematic representation of the four isoforms of SH2B1………………7

FIG. 1.2. Schematic of SH2B1β function in response to NGF in PC12 cells…..17

FIG. 2.1. Identification of SH2B1 mutations……………………………….…..22

FIG. 2.2. Expression profile of SH2B1 isoforms…………………………………25

FIG. 2.3. SH2B1β with human mutations do not enhance NGF-induced PC12 cell differentiation to the same extent as WT……………………..……………..……27

FIG. 2.4. All SH2B1β mutant proteins exhibited an impaired ability to accumulate in the nucleus…………………...…………………………………………………..28

FIG. 2.5. SH2B1β with human mutations inhibit GH-induced cell migration…....29

FIG. 2.6. SH2B1β P90H, P322S and A175N express at the appropriate size and to a similar extent as WT. They also exhibit similar enhancement of JAK2 phosphorylation...30

FIG. 2.7. SH2B1β truncation mutant F344LfsX20 expresses at the appropriate size but at a much lower level than WT. It does not enhance JAK2 phosphorylation…………………………………………………………………..32

FIG. 2.8. Subcellular distribution of SH2B1β WT and mutant proteins in 293T cells……………………………………………………………………...………33

FIG. 2.9. Subcellular distribution of SH2B1β WT and mutant proteins in PC12 cells…………………………………………………………………………...34

FIG. 2.10. SH2B1β mutants enhance leptin-induced tyrosyl phosphorylation of IRS2 to the same extent as SH2B1β WT………………………………………...35
FIG. 2.11. SH2B1β mutants enhance insulin-induced tyrosyl phosphorylation of IRS2 to the same extent as SH2B1β WT………………………………………...36
FIG. 2.12. Dimerization of SH2B1β with human mutations………………………37
FIG. 3.1. Identification of human mutations in SH2B1α…………………………...52
FIG. 3.2. SH2B1α point mutants but not F344LfsX20 enhance JAK2 activity…..54
FIG. 3.3. SH2B1α point mutants but not F344LfsX20 enhance insulin-induced IRS2 tyrosyl phosphorylation……………..…………………………………………..56
FIG. 3.4. SH2B1α does not enhance NGF-induced PC12 neurite outgrowth.….58
FIG. 3.5. SH2B1α does not enhance NGF-induced uPAR expression.…………59
FIG. 3.6. SH2B1α exhibits impaired ability to translocate to the nucleus………..61
FIG. 3.7. Subcellular distribution of SH2B1α WT and mutant proteins…………..62
FIG. 3.8. Subcellular distribution of SH2B1β and SH2B1α WT in response to serum……………………………………………………………………………...63
FIG. 3.9. SH2B1 1-620 enhances NGF-induced PC12 cell neurite outgrowth…65
FIG. 4.1. Schematic of SH2B1δ NLS mutations…………………………………...76
FIG. 4.2. ABCD. SH2B1δ requires NLS1 to localize at the PM and an intact bipartite NLS to localize at the nucleolus………………………………………...78
FIG. 4.2. EFG. SH2B1δ requires NLS1 to localize at the PM and an intact bipartite NLS to localize at the nucleolus……………………………………..…79
FIG. 4.2. IJK. SH2B1δ requires NLS1 to localize at the PM and an intact bipartite NLS to localize at the nucleolus…………………………………………...………80

FIG. 4.3. SH2B1δ WT, but not with a mutated NLS, enhances NGF-induced PC12 cell differentiation to the same extent as WT……………………………..82

FIG. 4.4. Identification of human mutations in SH2B1δ…………………………...85

FIG. 4.5. SH2B1δ with disease-causing human mutations do not enhance NGF-induced PC12 cell differentiation to the same extent as WT…………………..…86

FIG. 4.6. PC12 cells stably express GFP-SH2B1β, or GFP-SH2B1δ WT, P322S, or R680C……………………………………………………………………….….88

FIG. 4.7. SH2B1δ, but not SH2B1β, enhances NGF-induced ERK phosphorylation………………………………………..…………………...89

FIG. 4.8. SH2B1δ P322S and R680C enhance NGF-induced ERK phosphorylation to the same extent as SH2B1δ WT………………………………90

FIG. 4.9. Overexpression of SH2B1δ WT or with a human mutation promotes NGF-induced expression of MMP3 and MMP10 mRNA expression..................92

FIG. 4.10. SH2B1δ enhances JAK2 phosphorylation…………………………..…94

FIG. 4.11. SH2B1δ with a human mutation enhances JAK2 phosphorylation to the same extent as SH2B1δ WT……………………………………………………..95

FIG. 5.1. SH2B1 expression knocked-down by shRNA…………………….……...110

FIG. 5.2. Knock-down of SH2B1 decreases expression of 247 GH-dependent genes……………………………………………………………………………..…111

FIG. 5.3. Knock-down of SH2B1 decreases GH-dependent expression of Apelin and Cish………………………………………………………………….………113

FIG. 5.4. Reintroduction of SH2B1β rescues GH-dependent induction of Arid5a mRNA in shSH2B1 expressing cells…………………………………………..…115

FIG. 5.6. The NLS in SH2B1β is required for maximal GH-induced expression of Cish mRNA……………………………………………………………………………116

FIG. 6.1. Schematic of SH2B1 function in response to neurotrophic factors and where it is impaired by human mutations……………….…………………………132

List of Tables

Table 2.1. Phenotypic characteristics of SH2B1 mutation carriers……………...23

Table 6.1. Over-represented transcription factor binding sequence motifs…...129

Table 6.2. Identification of potential interacting partner of SH2B1……………..131

Table 6.3. Summary of assays presented in this dissertation by isoform of SH2B1…………………………………………………………………………....133

Abstract

EFFECTS OF HUMAN OBESITY-ASSOCIATED MUTATIONS ON CELLULAR ACTIONS OF THE ADAPTER PROTEIN SH2B1

By

Michael E. Doche

Chair: Christin Carter-Su

Src homology 2 B adapter protein 1 (SH2B1) modulates signaling by a variety of ligands that bind to receptor tyrosine kinases or JAK-associated cytokine receptors, including leptin, insulin, growth hormone (GH), and nerve growth factor (NGF). Targeted deletion of *Sh2b1* in mice results in increased food intake, obesity, and insulin resistance. SH2B1 is expressed as four different isoforms: alpha, beta, gamma, and delta. SH2B1 loss-of-function mutations were identified in a cohort of patients with severe early-onset obesity. Mutation carriers exhibit hyperphagia, childhood-onset obesity, disproportionate insulin resistance, and reduced final height as adults. Unexpectedly, mutation carriers also exhibit a spectrum of behavioral abnormalities that were not reported for

controls, including social isolation and aggression. The work in this thesis characterizes cellular functions of SH2B1 alpha, beta, and delta and examines how human mutations in *SH2B1* affect their functions. SH2B1beta with a human mutation exhibits an impaired ability to enhance NGF-induced neurite outgrowth, accumulate in the nucleus, and enhance GH-induced macrophage migration. In contrast to SH2B1beta, SH2B1alpha does not enhance NGF-induced neurite outgrowth or uPAR mRNA expression and does not cycle through the nucleus. SH2B1delta has a sub-cellular localization at the plasma membrane and nucleolus. A bipartite nuclear localization signal (NLS) in the unique C-terminal tail of SH2B1delta is important for its nucleolar localization and enhancement of NGF-induced neurite outgrowth. SH2B1delta with a disease-causing human mutation does not enhance NGF-induced neurite outgrowth to the same extent as SH2B1delta WT. Thus, the ability of SH2B1 isoforms to promote neuronal differentiation and cell migration may contribute to the control of human food intake and body weight and is implicated in maladaptive human behavior. To translate results from PC12 cells to a GH context, SH2B1, including its NLS region, was shown to be required for the enhancement of expression of a sub-set of GH-induced genes. Taken together, these results highlight the importance of understanding how the different isoforms of SH2B1 regulate gene transcription, cell motility, and neuronal differentiation to promote whole-body energy homeostasis and insulin sensitivity, and affect behavior.

Chapter 1

The Genetics of Obesity Study and Monogenic Causes of Obesity

Many years of a sedentary lifestyle along with over-consumption of high calorie foods are believed to be main factors contributing to a growing obesity epidemic in developed Western countries. However, these factors alone cannot be responsible for severe, early-onset obesity, or obesity seen in children (1). It has been thought that the main factors contributing to obesity in children must have a genetic component since children have not had the years of imbalanced energy intake to develop obesity as seen in adults. Many genetic consortiums have been established to identify genetic markers that may be contributing to human disease. One such consortium that was established in 1997 is the Genetics of Obesity Study (GOOS) (2). The aim of this consortium is to recruit patients with severe obesity [body mass index (BMI) sd score (SDS) > 3] of early onset (<10 years old), particularly children with a strong family history of obesity and those from consanguineous families. With the help of researchers and clinicians throughout the world, this consortium has to date recruited over 4500 patients to the GOOS cohort. In the past several years, GOOS and other research groups have described many human disorders of energy balance that

arise from genetic defects (3). All of these are in molecules identical or similar to those known to cause obesity in genetic and experimental syndromes of obesity in rodents, and all have been identified using a candidate gene approach. These mutations all result in severe obesity in childhood without developmental pleiotropic features. Many of the mutations that result in obesity encode genes for leptin, leptin receptor, or proteins involved in the transduction of the leptin signal in the central nervous system.

Leptin is an adipocyte-derived hormone that interacts with leptin receptors in neurons in the brain, particularly the arcuate nucleus and other regions of the hypothalamus, to influence whole-body energy homeostasis (4, 5). Leptin is found in the circulation at levels proportionate to body-fat content and acts to maintain energy balance by reducing appetite and increasing thermogenesis (6). When leptin signaling is disrupted, individuals do not feel sated after a meal and constantly seek food consumption. This pathological level of food intake is termed hyperphagia. Early studies have identified several individuals and families with mutations in the genes encoding leptin or the leptin receptor that suffer from obesity caused by hyperphagia (7-10).

Leptin receptor-expressing neurons project to groups of neurons within the hypothalamic arcuate nucleus that are critical to the regulation of food intake and energy balance. One group are pro-opiomelanocortin (POMC) neurons that produce α-MSH (melanocyte stimulating hormone) that is anorectic and another are neurons that express the orexigenic factors neuropeptide Y (NPY) and agouti-related protein (AGRP) (11). AGRP is an antagonist of MC3R and MC4R

(melanocortin 3 and 4 receptors), which act downstream of POMC neurons. Leptin regulates these neuronal populations in a reciprocal manner; it activates POMC neurons and inhibits NPY/AGRP neurons to decrease food intake and increase energy expenditure (12). Genetic variations that lead to deficiencies in *POMC* or *MC4R* expression or activity have been identified in patients with severe, early-onset obesity caused by hyperphagia (reviewed in (3) and (2)). Additional mutations in children with severe obesity have been identified in the genes encoding brain-derived neurotrophic factor (BDNF) and its receptor, tyrosine kinase TrkB (13-15). BDNF is important for development, survival, and differentiation of neurons (16). Recently, investigators have seen that leptin-stimulated neural activity induces dendritic BDNF synthesis, which is important for transducing the neural leptin signal required to promote nutritional satiety (17). Thus, human mutations that disrupt the neuro-endocrine signaling of leptin cause hyperphagic obesity.

The adapter protein SH2B1 (which will be discussed more thoroughly later in this report) is important for mediating leptin signal transduction (18, 19). The long form of the leptin receptor interacts with the receptor-associated tyrosine kinase JAK2 to initiate intracellular signal transduction (20, 21). SH2B1 has been reported to interact with and potentiate the activation of JAK2 (22, 23). In response to leptin, SH2B1 binds to phospho-tyrosine 813 in JAK2 and not only enhances total JAK2 activity (which globally enhances the leptin signal) but also enhances recruitment insulin receptor substrate 1 (IRS1) to be phosphorylated by JAK2 and activate the downstream phosphatidylinositol 3-kinase (PI3K)

pathway (19). This evidence shows that SH2B1 is an important positive regulator of leptin signal transduction. As with mutations in other genes that mediate the leptin signal, dysfunction-causing mutations in *SH2B1* would be expected to lead to leptin resistance and hyperphagic obesity in humans. The effects on the cellular actions of SH2B1 of human obesity-associated mutations identified in *SH2B1* will be discussed in this dissertation.

The Adapter Protein SH2B1

Adapter proteins are important signaling molecules that usually do not have enzymatic capabilities of their own, but rather function to link enzymatic proteins with their substrates to facilitate the enzymatic reaction. Adapter proteins play a role in signal transduction from ligand-stimulated receptor or receptor-associated tyrosine kinases. Ligand binding activates tyrosine kinase enzymatic activity producing phospho-tyrosine residues that can serve as docking sites for proteins with Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (24). Adapter proteins usually consist of multiple protein-protein interaction domains (including SH2 or PTB domains) to link signaling molecules in a complex to facilitate the transduction of the extracellular signal. One group of adapter proteins that play a role in various signaling pathways is the SH2B family. There are three members of the SH2B family: SH2B1 (formerly named SH2-B, also known as PSM), SH2B2 (formerly named APS) and SH2B3 (formerly named Lnk). These proteins share a common domain structure consisting of a dimerization domain, proline rich domains, a PH domain and a SH2 domain and were first identified as proteins involved in immune cell activation signaling (25-27). There have been four SH2B1 isoforms, produced by variable mRNA splicing, identified. The four isoforms, termed alpha, beta,

gamma, and delta, are identical in sequence except for the region downstream of the SH2 domain splice sites. Variable mRNA splicing confers unique C-terminal sequences for each isoform (28, 29) (FIG. 1.1).

SH2B1 has been identified as having a role in the signal transduction process for several receptor tyrosine kinases, including the receptors for NGF (30, 31), insulin (32, 33), insulin-like growth factor I (IGF-I)(34), brain-derived neurotrophic factor (BDNF) (30), glial cell line-derived neurotrophic factor (GDNF)(35), platelet-derived growth factor (PDGF)(36), and fibroblast growth factor (FGF)(37). SH2B1 has also been seen to play role in signaling for the JAK family of receptor-associated tyrosine kinases (22, 23, 38).

The mechanism by which SH2B1 interacts with JAK2 and mediates its signal transduction has been investigated in detail. SH2B1 acts to enhance JAK2 activation and autophosphorylation while also recruiting proteins to active JAK2 to mediate activation of signaling pathways. One proposed mechanism of how SH2B1 enhances JAK2 activity is that dimerization of SH2B1 leads to dimerization of its associated JAK2, which then enhances JAK2 activity (38). Another proposed mechanism is that SH2B1 binds to an already active (and autophosphorylated) JAK2 to cause a conformational change that maintains JAK2 in an active state (39). The latter is supported by the results that only the SH2 domain of SH2B1β and only one SH2B1β needs to be bound to a JAK2 dimer to increase the activity of JAK2. Both models agree, though, that SH2B1 directly functions to increase JAK2 activity and does not compete with an

inhibitor or recruit an activator to potentiate JAK2 activity. It has been shown that

SH2B1β

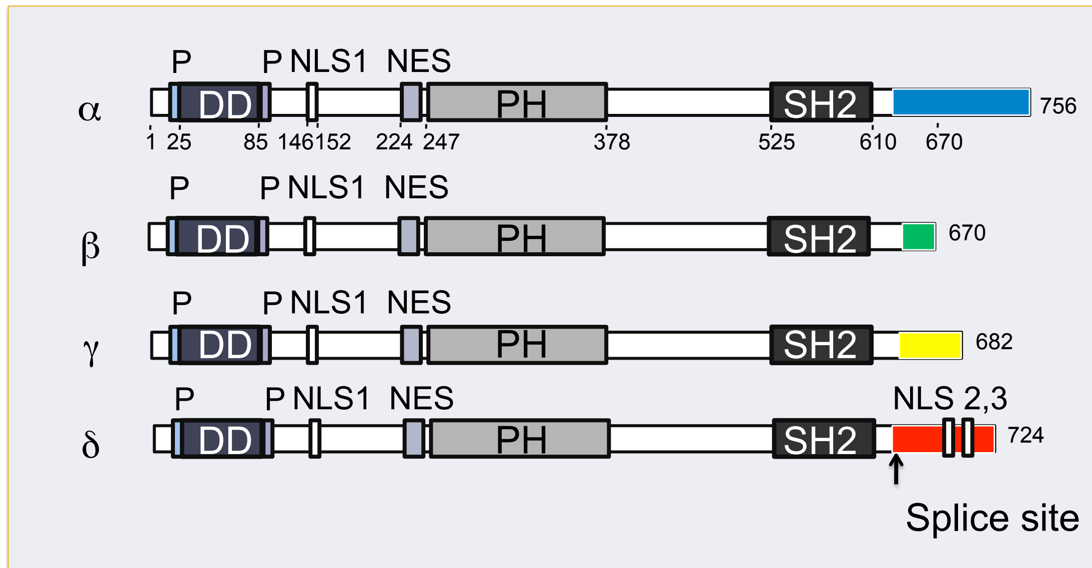


FIG. 1.1. Schematic representation of the four isoforms of SH2B1. P = Proline-rich region, DD = Dimerization Domain, NLS = Nuclear Localization Sequence, NES = Nuclear Export Sequence, PH = Plextrin homology, SH2 = Src Homology 2 domain, arrow = Variant splice site. Isoform-specific C-terminal regions indicated by unique color.

binds to phospho-tyrosine 813 that lies in an YXXL motif in the pseudokinase domain (JH2) of JAK2 (40). The JH2 domain is thought to be an autoinhibitory region of JAK2 that blocks the JH1 kinase domain and becomes displaced upon ligand binding to allow for JH1 kinase domain activity to the associated receptor (41). JAK2 has also been seen to phosphorylate SH2B1β on tyrosines 439 and 494 (42). Growth hormone (GH) is able to stimulate actin cytoskeletal rearrangement, membrane ruffling, and cell motility. SH2B1β WT, but not SH2B1β with mutated tyrosines 439 and 494, is able to potentiate this effect (42-44). SH2B1β is thought to elicit this effect at least in part by recruiting the actin cytoskeleton regulator Rac to GH-activated JAK2/GH receptor complexes. The effects of GH on actin dynamics and cell motility are blunted when SH2B1 cannot interact with Rac (45). Thus, SH2B1 acts to enhance signal transduction from JAK2 by binding to JAK2 and maintaining it in a more active confirmation and by recruiting effector proteins to active JAK2 at the plasma membrane.

The role of SH2B1 in insulin signaling has been investigated *in vitro* and *in vivo*. SH2B1 binds to phospho-tyrosines in the activation loop of the insulin receptor (IR) via its SH2 domain and is tyrosyl phosphorylated (32, 33, 46, 47). SH2B1 enhances IR activity and cellular responses, including mitogenesis and glucose uptake (48, 49). Expression of SH2B1 stimulates IR activity leading to increased IRS phosphorylation and PI3K signaling, as well as extracellular regulated kinase (ERK) signaling (50, 51). SH2B1 expression delays dephosphorylation of IR and IRS (50) and protects IRS from phosphatases (51). In mice, deletion of *Sh2b1* impairs insulin receptor activation and signaling in the

liver, skeletal muscle, and adipose tissue leading to the development of hyperinsulinemia, hyperglycemia, and glucose intolerance (52, 53). Thus, SH2B1 has been implicated as an important mediator of insulin signaling and is required for maintaining normal insulin sensitivity and glucose homeostasis.

SH2B1 has been implicated in the regulation of whole-body energy homeostasis. The SH2B1$^{-/-}$ mice generated by Ren et al. display hyperphagia and obesity (18). Plasma leptin levels in these mice were elevated before the onset of obesity, suggesting that systemic leptin resistance is a primary contributing factor to the obesity rather than a symptom. As mentioned previously, SH2B1$^{-/-}$ mice displayed age-dependent glucose intolerance, hyperglycemia, and hyperinsulinemia (52), consistent with SH2B1 being a positive regulator of insulin signaling. However, when SH2B1 is transgenically expressed only in neurons in SH2B1$^{-/-}$ mice, the hyperleptinemia, obesity, and insulin resistance are rescued (54). This phenotype suggests that only neuronal SH2B1 is required to reverse the metabolic disorder seen in SH2B1-whole body KOs. A second group has also generated SH2B1$^{-/-}$ mice but did not report development of an obese phenotype (55). They concluded that their SH2B1$^{-/-}$ mice remained lean because SH2B1 is a critical positive regulator of adipogenesis by mediating insulin/IGF-1 signaling (56). It is hypothesized that differences in diet and environmental stress can possibly account for the discrepancies in phenotype (18). However, both groups shared findings of infertility and neonatal growth retardation between 2-6 weeks of birth, consistent with SH2B1 playing a role in GH and IGF-1 signaling.

There has been a growing body of evidence implicating a role for SH2B1 in neurotrophic function, including neuronal differentiation and TrkA signaling. Yeast two-hybrid screens identified SH2B1 (later identified to be the gamma isoform by unpublished observation from the Carter-Su Lab) and SH2B2 as interacting partners of TrkA via phosphorylated tyrosines in the kinase activation loop (30). GST pull down assays confirmed the phospho-tyrosine dependent interaction between SH2B1β and TrkA as the interaction was only seen using cells that had been treated with NGF. It was not seen when SH2B1β with an inactivating mutation in the SH2 domain was employed (31), suggesting that the interaction with TrkA requires an intact SH2 domain. SH2B1β becomes tyrosyl phosphorylated in response to NGF and is recruited to active TrkA. Similarly, Shc and phospholipase C (PLC)-γ are also recruited to activated TrkA and phosphorylated in response to NGF (30, 31, 57-59). While TrkA phosphorylation of PLC-γ initiates its enzymatic activity (60), tyrosyl phosphorylation of SH2B1 is assumed to create docking sites for protein complex formation at the receptor to mediate signal transduction cascades, similar to the adapter protein Shc when tyrosyl phosphorylated by active TrkA. Tyrosyl phosphorylated Shc recruits Grb2-SOS complexes which leads to the activation of the Ras-ERK signaling cascade (59, 61). SH2B1 has also been seen to interact with Grb2 (30), suggesting SH2B1 may play a similar role as Shc in NGF signaling.

SH2B1 promotes neuronal survival and differentiation. Rat neonatal sympathetic neurons cultured with NGF had greatly reduced survival rates when anti-SH2B1 antibodies were introduced intracellularly to reduce the activity of

endogenous SH2B1. Sympathetic neurons also exhibited axonal degeneration when transfected with cDNA encoding a truncated SH2B1 mutant. In contrast, overexpression of cDNA encoding SH2B1 WT promoted long, branched axonal outgrowths (30). Overexpression of SH2B1 enhances NGF-induced neurite outgrowth in PC12 cells (the sympathetic neuron-like PC12 cell line will be described later in this chapter), while shRNA-mediated knockdown of endogenous SH2B1 significantly decreased NGF-induced neurite outgrowth (62). Partially conflicting mechanisms by which SH2B1 mediates these effects have been reported, which may be a result of which SH2B1 isoform was used in the investigations. Overexpression of SH2B1γ in PC12 cells led to heightened and prolonged TrkA tyrosine phosphorylation as well as heightened and prolonged phosphorylation of ERKs 1/2 (63). Qian et al. concluded that SH2B1 promotes neuronal differentiation as a consequence of increasing the activity of TrkA and the ability of SH2B1 to recruit Grb2 to initiate Ras-dependent signaling, leading to increased and prolonged ERK 1/2 phosphorylation levels. However, other evidence suggests that SH2B1β may promote neuronal differentiation through a different mechanism. While SH2B1β enhanced and SH2B1β(R555E) inhibited neuronal differentiation in PC12 cells, neither was seen to alter NGF-induced TrkA autophosphorylation or phosphorylation of downstream signaling proteins like ERKs 1/2 (31). These results suggest that SH2B1β may not be directly affecting TrkA activation and could be regulating NGF-induced neuronal differentiation through a pathway parallel or downstream of ERKs 1/2.

SH2B1 plays a role in mediating signaling for other neurotrophic factors such as BDNF and GDNF. SH2B1 binds to active TrkB and is phosphorylated in response to BDNF (30, 64). SH2B1 also interacts with RET, the receptor for GDNF, and facilitates GDNF-induced neurite outgrowth in PC12 cells and mesencephalic neurons (35). These findings implicate SH2B1 in having a broader role in neurotrophic signaling and neuronal development.

While investigating the mechanism by which SH2B1β enhances NGF-induced neuronal differentiation, it was discovered that SH2B1β is capable of localizing in the cell nucleus. Although microscopy images show a steady-state localization of SH2B1β WT at the PM or cytosol (65), inhibition of Crm1-mediated nuclear export with leptomycin B caused SH2B1β to accumulate in the nucleus (66). Chen et al. identified a nuclear-export sequence (NES) in SH2B1 that when mutated or deleted also causes SH2B1β to accumulate in the nucleus (66). SH2B1β without a functional NES is unable to enhance NGF-induced PC12 cell differentiation (66). Further investigation identified a nuclear localization signal (NLS) in SH2B1β and mutation of this NLS also abolishes the ability of SH2B1β to enhance NGF-induced neuronal differentiation in PC12 cells (62). The NLS of SH2B1β, along with the dimerization domain, has also been shown to be required for SH2B1β to localize to the PM (65). Phosphorylation of serine residues proximal to the NLS (primarily Ser161 and Ser165) is necessary for SH2B1β to be released from the PM and translocate to the nucleus to enhance NGF-induced neurite outgrowth (65). These data suggest that while SH2B1β has a steady-state sub-cellular localization at the PM and cytosol, it is

constitutively shuttling in and out of the nucleus. Further, this nucleo-cytoplasmic cycling is required for SH2B1β to be able to enhance NGF-induced neuronal differentiation.

Subsequent investigation into the nuclear role of SH2B1 in NGF-induced neuronal differentiation was conducted. Microarray analysis of NGF-treated (100 ng/ml for 6 hours) PC12 cells expressing SH2B1β WT or the dominant-negative SH2B1β (R555E) revealed a sub-set of NGF-induced genes that require SH2B1β for their full NGF-induced expression (67). Among this sub-set, some of the most highly NGF and SH2B1β regulated genes were *Plaur*, *Mmp3*, and *Mmp10*, which encode the proteins urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 3 (MMP3), and MMP10, respectively. These proteins are involved in the same extracellular matrix degradation pathway important for neurite outgrowth in differentiating neurons (68-71). In this pathway, uPAR binds the inactive proform of urokinase plasminogen activator (pro-uPA), allowing it to be cleaved by cathepsins and become activated. The activated uPA proteinase then cleaves inactive plasminogen to form enzymatically active plasmin, which in turn cleaves inactive pro-MMPs to form 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 (72) and (73)). It has also been suggested that uPAR is an immediate-early gene product of NGF whose function may be required for induction of secondary response genes important for differentiation, such as *Mmp3* (69, 74). In other cell types, uPAR has been reported to activate a variety of intracellular signaling pathways,

including JAK1/signal transducer and activator of transcription (STAT) 1 (75), MEK/ERK (76), the Src family kinase hck (77), and protein kinase Cε (78) pathways, as well as increase levels of signaling molecules including diacylglycerol (79, 80), cAMP (81), calcium released from internal stores (82), and inositol phosphate turnover (83). uPAR has also been reported to form stable complexes with integrins that alter the adhesive properties of cells (84) (85). Expression of SH2B1β with a mutant NLS does not promote the NGF-induced expression of these genes, possibly explaining the lack of enhancement in NGF-induced neuronal differentiation seen for PC12 cells expressing SH2B1β with a mutant NLS.

PC12 Cells: Cell Culture Model of Neuronal Differentiation

The PC12 cell line has been commonly used as a model system to investigate the mechanisms of NGF-mediated neuronal differentiation and NGF signal transduction (86). PC12 cells are derived from a solid rat pheochromocytoma tumor and, when treated with NGF, differentiate into a neuronal type cell with properties similar to those of sympathetic neurons (87). When PC12 cells are treated with NGF, they stop proliferating and initiate a differentiation process in which they exhibit cellular hypertrophy, produce neurite outgrowths, express neuronal-specific markers, and become electrically excitable (86, 87). Differentiated sympathetic-like PC12 neuronal cells are able to form synapses with primary neurons (88), supporting the biological relevance of this *in vitro* cell line.

The PC12 cell model system has been used to elucidate the molecular pathways regulating the transformation to a mature neuron. Upon NGF binding to TrkA, a series of post-translational protein modifications and differential gene expression leads to the biochemical and morphological cellular changes that contribute to the differentiated neuronal state. NGF stimulation activates multiple signaling pathways including PI3K/Akt, PLC-γ, Ras/Raf/MEK/ERKs 1 and 2, Jun N-terminal kinase (JNK), p38, and atypical protein kinase Cs (89, 90). Epidermal

growth factor (EGF), while promoting the opposite biological response of proliferation compared to differentiation, promotes the activation of some of the same signaling pathways in PC12 cells as NGF (91). Whereas NGF-activated TrkA ceases cell division and promotes a differentiation protocol, activated EGF receptor (EGFR) promotes cellular proliferation (92). Research into how activation of the same molecular pathways can lead to different biological responses has revealed that the duration of ERK activation is a primary factor responsible for the different responses. A transient or brief activation of ERKs by EGF promotes mitogenesis while a more sustained activation in response to NGF promotes neurogenesis (93-96). It has since been shown that ERK activation is required for NGF-induced PC12 cell neuronal differentiation (97, 98). It appears that the difference in the duration of ERK activation in response to NGF and EGF is due to differential recruitment of adaptor proteins to the active receptors. Activated TrkA, but not EGFR, recruits a complex of proteins including the adaptor protein FRS2, Crk, C3G, Rap1 and B-Raf, which results in sustained ERK 1/2 activation (99, 100). These works highlight the importance of understanding the roles of adaptor proteins in mediating the cellular responses to activated TrkA.

A schematic summarizing the functions of SH2B1β in response to NGF is depicted in FIG. 1.2.

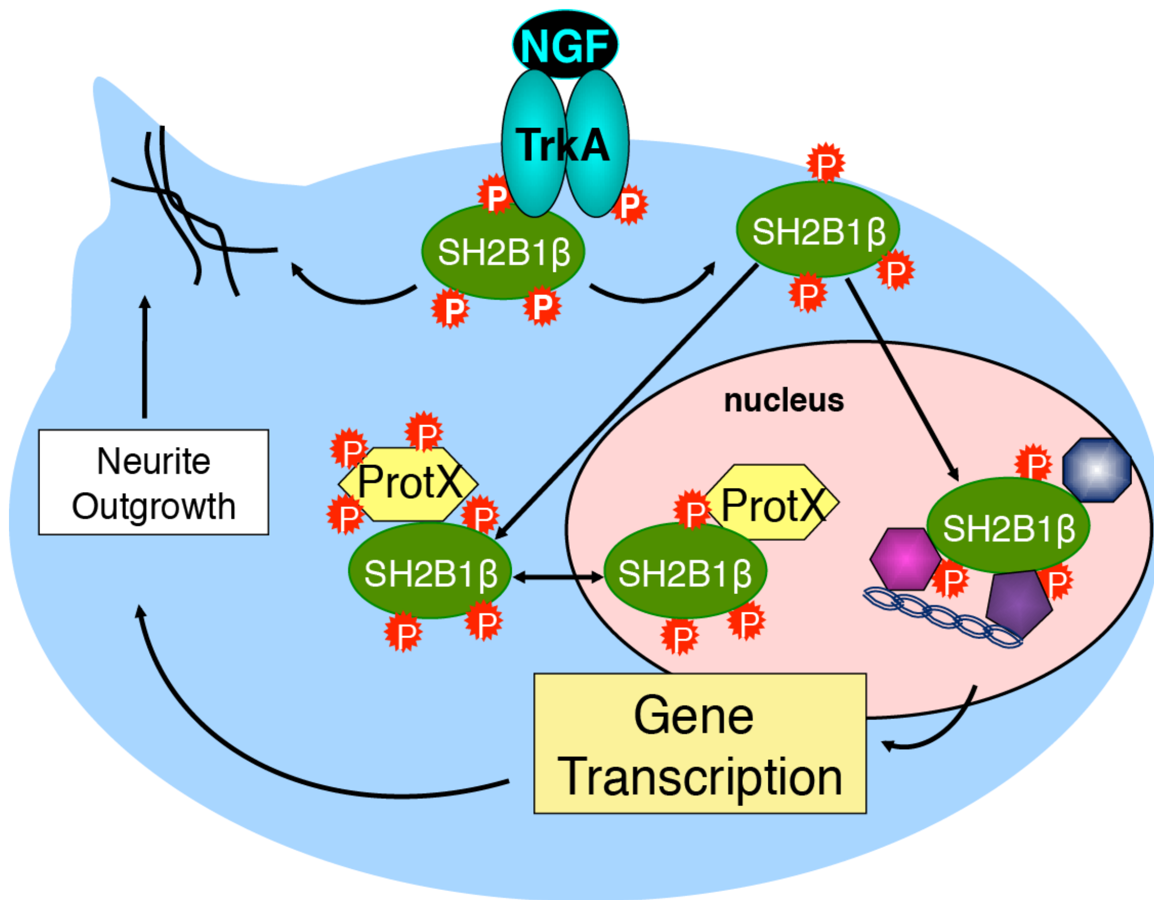


FIG. 1.2. Schematic of SH2B1β function in response to NGF in PC12 cells. SH2B1β localizes at the plasma membrane and cytosol and cycles in and out of the nucleus. SH2B1β acts to enhance NGF-induced gene expression and neurite outgrowth. It is hypothesized that SH2B1β acts to form a transcription-activating complex in the nucleus or promotes gene transcription by shuttling a repressor transcription factor out of the nucleus or a signaling molecule into the nucleus (ProtX).

Chapter 2

Human *SH2B1* mutations are associated with maladaptive behaviors and obesity

Abstract

Src homology 2 B adapter protein 1 (SH2B1) modulates signaling by a variety of ligands that bind to receptor tyrosine kinases or JAK-associated cytokine receptors, including leptin, insulin, growth hormone (GH), and nerve growth factor (NGF). Targeted deletion of *Sh2b1* in mice results in increased food intake, obesity, and insulin resistance, with an intermediate phenotype seen in heterozygous null mice on a high-fat diet. We identified SH2B1 loss-of-function mutations in a large cohort of patients with severe early-onset obesity. Mutation carriers exhibited hyperphagia, childhood-onset obesity, disproportionate insulin resistance, and reduced final height as adults. Unexpectedly, mutation carriers exhibited a spectrum of behavioral abnormalities that were not reported in controls, including social isolation and aggression. We conclude that SH2B1 plays a critical role in the control of human food intake and body weight and is implicated in maladaptive human behavior.

Introduction

Leptin is a 16-kDa circulating hormone that regulates energy homeostasis via hypothalamic neurons expressing the leptin receptor (LEPR) (101). Congenital deficiency of leptin and its receptor results in severe obesity in rodents and humans, implicating leptin-mediated signaling in the regulation of food intake, energy expenditure, carbohydrate metabolism, and neuroendocrine function (10, 102). Diet-induced obesity in rodents, and common forms of obesity in humans, are characterized by resistance to endogenous and exogenous leptin (103). Dissecting the intracellular signaling pathways and hypothalamic neural circuitry by which leptin exerts its effects is critical for the identification of potential therapeutic targets for obesity (104).

Leptin mediates effects on energy balance by binding to the long form of LEPR (LEPRb) and activating LEPRb-associated JAK2 (105). JAK2 phosphorylates multiple tyrosines in LEPRb (Tyr985/1107/1138), enabling recruitment of downstream effectors. JAK2 also autophosphorylates on Tyr813, allowing the binding of Src homology 2 (SH2) B adapter protein 1 (SH2B1), which enhances JAK2 activation and helps recruit insulin receptor substrate (IRS)1 and IRS2 to the LEPRb/JAK2 complex (18, 40). This facilitates JAK2-mediated tyrosine phosphorylation of IRS1/2 and subsequent activation of the PI3K pathway.

SH2B1 is a key endogenous positive regulator of leptin sensitivity; targeted deletion in mice results in impaired leptin signaling and severe obesity (18). *Sh2b1*-null mice are also insulin resistant and exhibit impaired insulin signaling (51). Multiple isoforms of SH2B1 are expressed in the brain; however, neuron-specific restoration of recombinant *Sh2b1β* alone is sufficient to reverse the obesity observed in *Sh2b1* knockout mice (54), which suggests that centrally expressed SH2B1β is critical to energy homeostasis.

To investigate the importance of SH2B1 in human energy homeostasis and insulin signaling, we sought to identify mutations in the *SH2B1* gene in a group of severely obese patients. We identified 300 patients with severe early-onset obesity from the Genetics of Obesity Study (GOOS) cohort (106) with a disproportionate degree of insulin resistance for their obesity, as defined by the presence of acanthosis nigricans, development of type 2 diabetes in early adolescence, and/or markedly elevated plasma insulin (top decile for age, gender, and BMI). Mutations in the genes causing the known monogenic obesity syndromes had been excluded in these patients, as had deletions at 16p11.2 by multiplex ligation–dependent probe amplification (MLPA) (107).

Results and Discussion

We identified 5 probands of mixed European descent with heterozygous mutations in SH2B1 (FIG. 2.1): a frameshift mutation, F344LfsX20, which leads to a truncated protein product, and 3 missense mutations, P90H (2 patients), T175N, and P322S; all were absent from 500 control subjects ($P < 0.001$). Probands were apparently unrelated over 3 generations, as assessed by medical history.

All mutations were inherited from overweight/obese parents, and carriers were hyperphagic and had reduced final height as adults (Table 2.1). Mutation carriers were hyperinsulinemic (fasting plasma insulin >60 pmol/l) and euglycemic; liver function tests and lipid profiles were within the normal range (data not shown). Unexpectedly, we found that mutation carriers were reported to have delayed speech and language development and aggressive behavior by healthcare professionals and by family members (Table 2.1). However, these individuals did not consent to further behavioral testing, so the precise nature and severity of these phenotypes could not be determined. None of the controls were reported to have behavioral abnormalities by healthcare professionals.

We next sought to assess the molecular and cellular basis for the phenotypes associated with these human mutations. SH2B1 is a member of a family of SH2 domain–containing adaptor molecules — SH2B1 (also known as

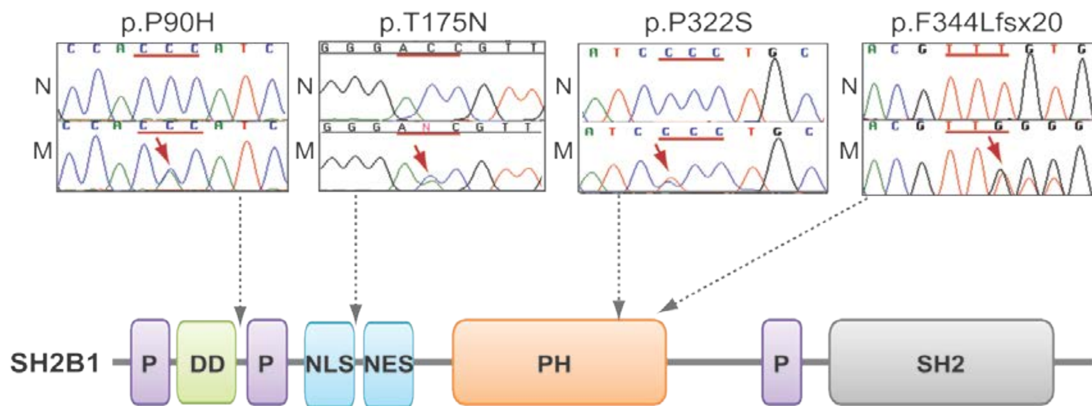


FIG. 2.1. Identification of SH2B1 mutations. Human SH2B1 protein (NP_001139268). P, proline-rich region; DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain. SH2B1 mutations are shown, with nucleotide changes marked on the chromatograms (arrows). N, normal allele; M, mutated allele.

Table 2.1. Phenotypic characteristics of SH2B1 mutation carriers. Probands were identified by genetic screening; other individuals are family members of probands as indicated. Data were only included where reports from healthcare professionals and family members were available. –, data missing (in some cases, family members were deceased or contact information was not available); N, normal allele; M, mutant allele.

	Mutation	Age	BMI (SD score)	M/F	Geno-type	Neurobehavioral Phenotypes
proband	P90H	27	45	M	NM	Social isolation, aggression
proband	T175N	4	29 (5.1)	F	NM	Speech and language delay, aggression
mother	T175N	31	30	F	NM	Speech and language delay
father	T175N	30	29	M	NN	Nil
sister	T175N	7	21 (2.2)	F	NM	Speech and language delay
proband	P322S	14	32 (2.9)	M	NM	Social isolation, aggression
mother	P322S	54	27	F	NM	Nil
father	P322S	55	22	M	NN	Nil
brother	P322S	18	32	M	NM	Social isolation
proband	F344LfsX20	13	37 (3.5)	F	NM	Social isolation, aggression
mother	F344LfsX20	48	48	F	NM	Social isolation
father	F344LfsX20	55	26	M	NN	Nil
sister	F344LfsX20	21	-	F	NN	Nil

PSM), SH2B2 (also known as APS), and SH2B3 (also known as Lnk) — that bind to activated receptor tyrosine kinases, including insulin receptor and TrkA, the receptor for nerve growth factor (NGF) (39). SH2B1 can also bind to activated JAK2 (108), a cytokine receptor–associated tyrosine kinase that is activated after binding of cytokine receptor ligands, such as leptin and growth hormone (GH). SH2B1 has an amino-terminal dimerization domain, nuclear localization and export sequences, a central pleckstrin homology domain, and a carboxy-terminal SH2 domain (FIG. 2.1).

The SH2B1 transcript undergoes alternative splicing at the 3′ end, giving rise to four protein products (SH2B1α, SH2B1β, SH2B1γ, and SH2B1δ; ref. (28)) that share their amino termini, nuclear localization sequence, nuclear export sequence, pleckstrin homology domain, and SH2 domain, but differ at their carboxyl termini. We investigated expression of the four SH2B1 isoforms in human tissues by RT-PCR (FIG. 2.2). As the beta isoform is the predominant form in the hypothalamus, we introduced the mutations into SH2B1β and examined their effect on SH2B1β expression, subcellular localization, and ability to enhance NGF-induced neuronal differentiation, cycling through the nucleus, GH-induced macrophage motility, JAK2 activation, leptin signaling, and insulin signaling.

Previous studies implicated SH2B1 in neuronal differentiation induced by NGF or by glial cell line–derived neurotrophic factor (30, 35, 109). To test the mutations’ effect on the ability of SH2B1β to enhance neuronal differentiation, we transiently expressed GFP-

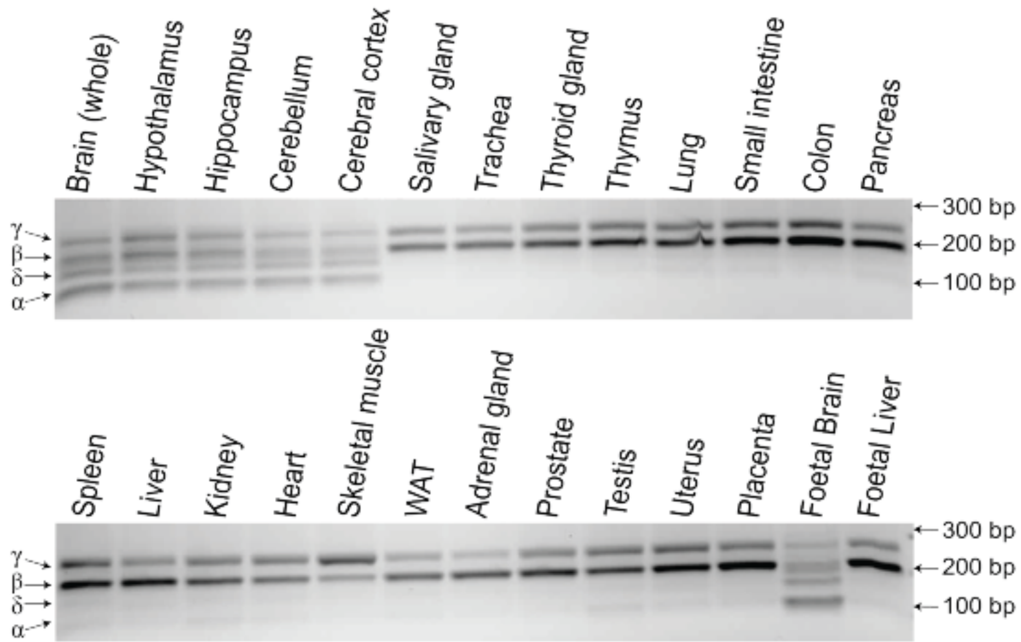


FIG. 2.2. Expression profile of SH2B1 isoforms. Expression of the 4 SH2B1 isoforms (α, β, γ, and δ), studied by qualitative RT-PCR in a panel of human tissues.

tagged WT or mutant SH2B1β in PC12 cells, treated the cells with NGF to induce neuronal differentiation, and determined the percentage of GFP+ cells that were differentiated (neurite outgrowths >2 times the length of the cell body). The truncation mutation failed to enhance NGF-induced neuronal differentiation, and the P90H, A175N, and P322S mutations significantly impaired the ability of SH2B1β to enhance neuronal differentiation compared with WT (FIG. 2.3).

Nuclear shuttling of SH2B1β appears to be required for its stimulatory effect on neuronal differentiation (62). To test whether the mutations impair the ability of SH2B1β to translocate to the nucleus, we treated 293T cells expressing GFP-tagged forms of SH2B1β with an inhibitor of nuclear export, leptomycin B, for 8 hours. While confocal microscopy revealed that approximately 95% of the cells expressing GFP-tagged WT SH2B1β showed a nuclear/cytoplasmic GFP fluorescence ratio of ≥ 1, cycling of the mutants into the nucleus was significantly impaired (FIG. 2.4).

SH2B1 has been implicated in GH regulation of the actin cytoskeleton (45). We therefore examined the ability of WT and mutant forms of SH2B1β to enhance GH-induced motility of cultured RAW264.7 macrophages. In contrast to GFP-tagged WT SH2B1β, which stimulated both basal and GH-induced motility, the point mutants inhibited GH-induced motility (FIG. 2.5).

SH2B1β mutants P90H, A175N, and P322S were expressed at the appropriate size and intensities and retained their ability to activate JAK2 (FIG. 2.6). In contrast, the F344LfsX20 mutant exhibited significantly reduced

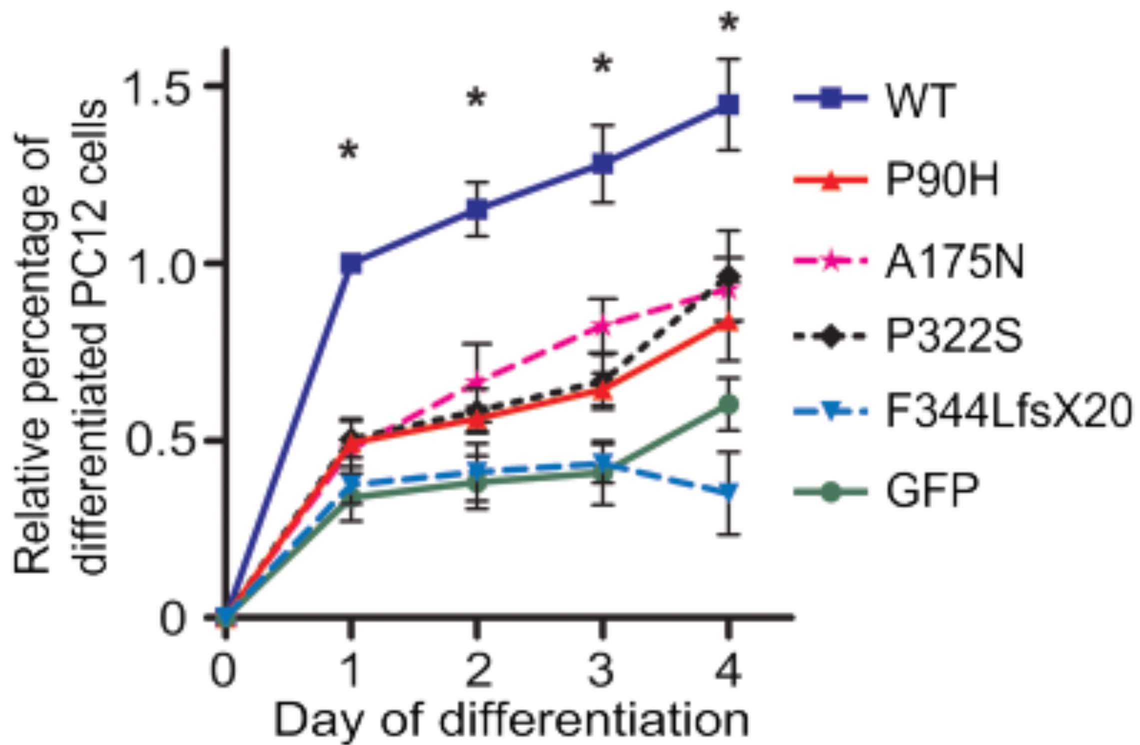
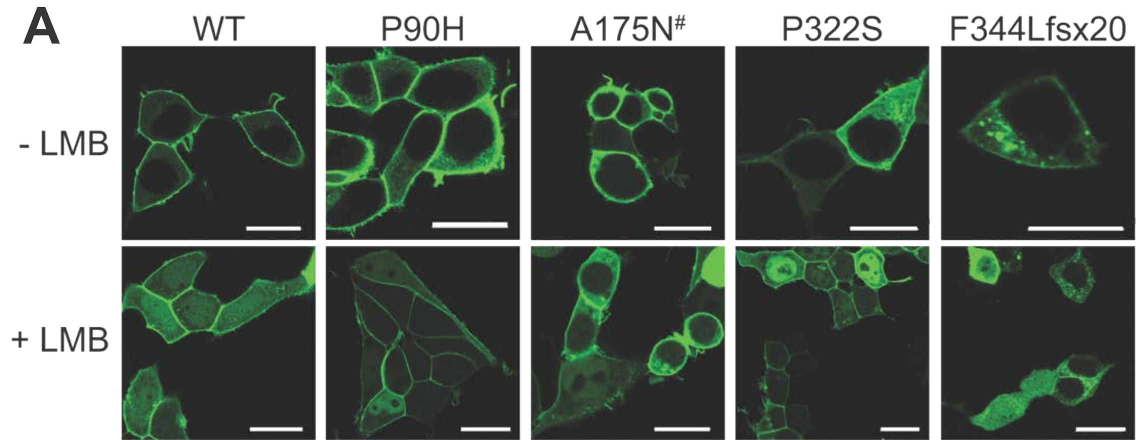


FIG. 2.3. SH2B1β with human mutations do not enhance NGF-induced PC12 cell differentiation to the same extent as WT. PC12 cells transiently expressing GFP-tagged WT or mutant SH2B1β were treated with NGF to induce differentiation. Percent differentiated values were normalized against WT at day 1 of differentiation (means ± SEM). All mutants impaired the rate of NGF-induced neuronal differentiation compared with WT. *P < 0.0001, 1-way ANOVA with Dunnett's post-test.



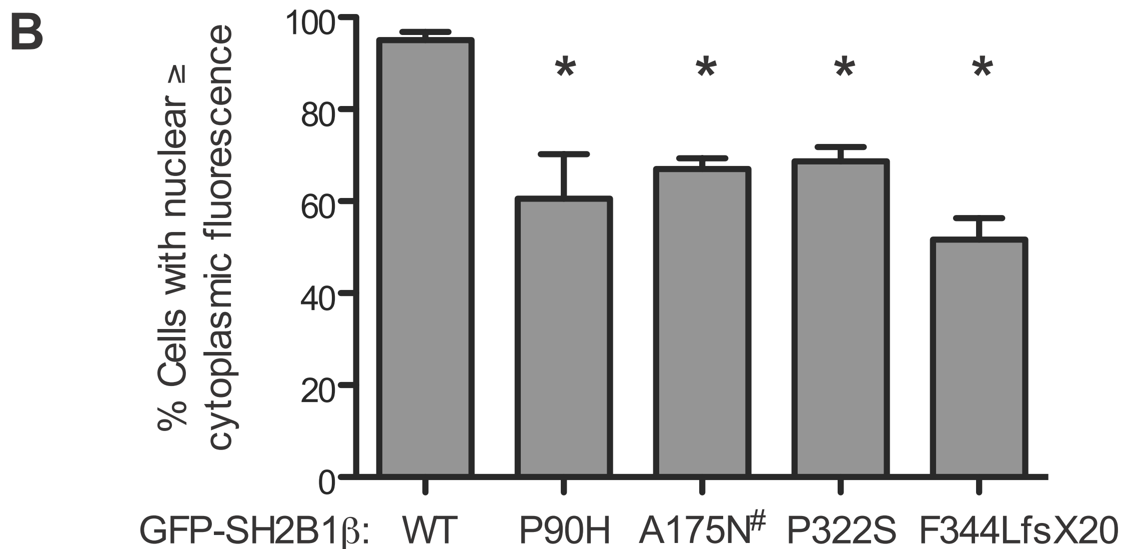


FIG. 2.4. All SH2B1β mutant proteins exhibited an impaired ability to accumulate in the nucleus. (**A**) 293T cells transiently expressing GFP-tagged WT or mutant SH2B1β were treated with or without leptomycin B (LMB) (20mM) for 8 h and imaged using fluorescent confocal microscopy. Scale bars: 10 μm. (**B**) The percentage of GFP-positive cells (76-150 cells/condition/assay) that exhibited nuclear fluorescence signal intensity greater than or equal to the cytoplasmic fluorescence signal intensity was determined. Means ± SEM are shown where n = 5, 4 and 3 independent experiments for WT, point mutants and F344LfsX20, respectively. (*) $p<0.05$ compared to WT using an unpaired, two-tailed Student's t test. p = 0.0128, 0.0004, 0.0001 and 0.0062 for P90H, P322S, A175N and F344LfsX20, respectively. (#) All experiments were performed using rat SH2B1β where Thr175 is Ala175 and therefore the T175N human mutation is referred to as A175N.

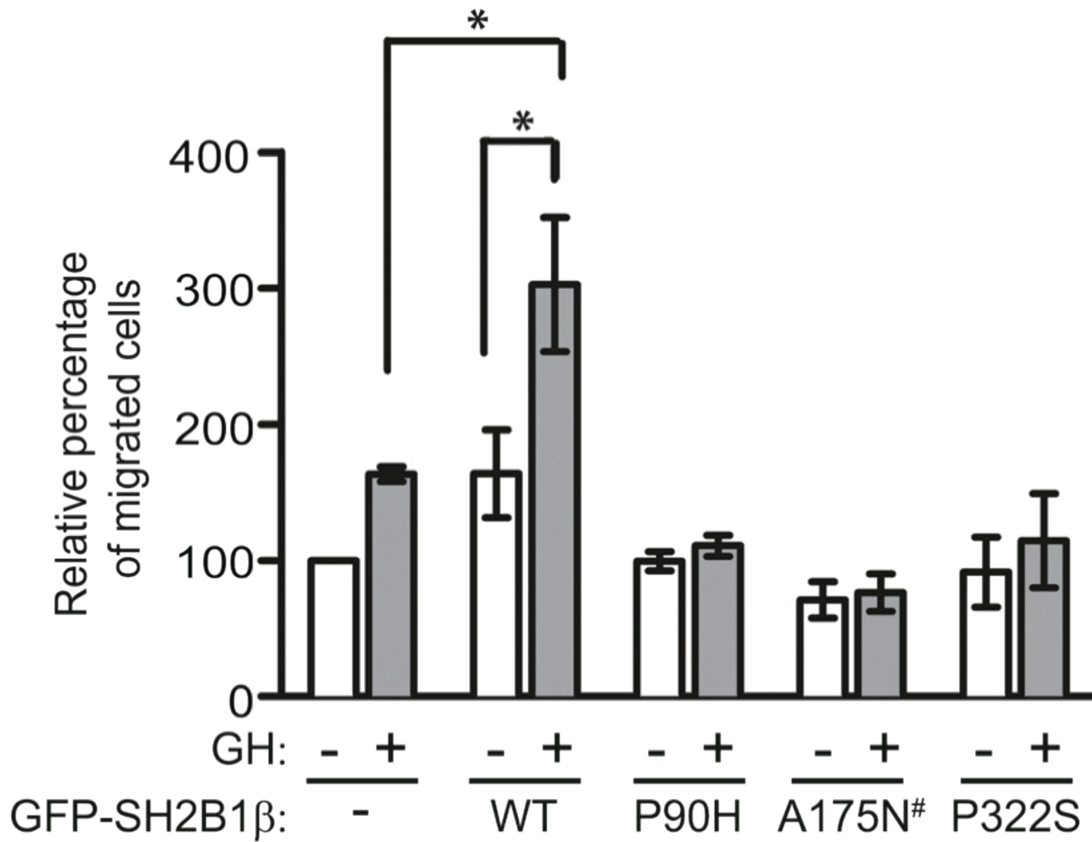


FIG. 2.5. SH2B1β with human mutations inhibit GH-induced cell migration. RAW264.7 macrophages transiently expressing GFP-tagged WT or mutant SH2B1β were added to the upper chamber, and GH (500 ng/ml) to the lower chamber, of a Transwell plate. Average values for migrated cells were normalized to unstimulated control values (means ± SEM). All SH2B1β mutants inhibited GH-induced cell migration. *$P < 0.05$.

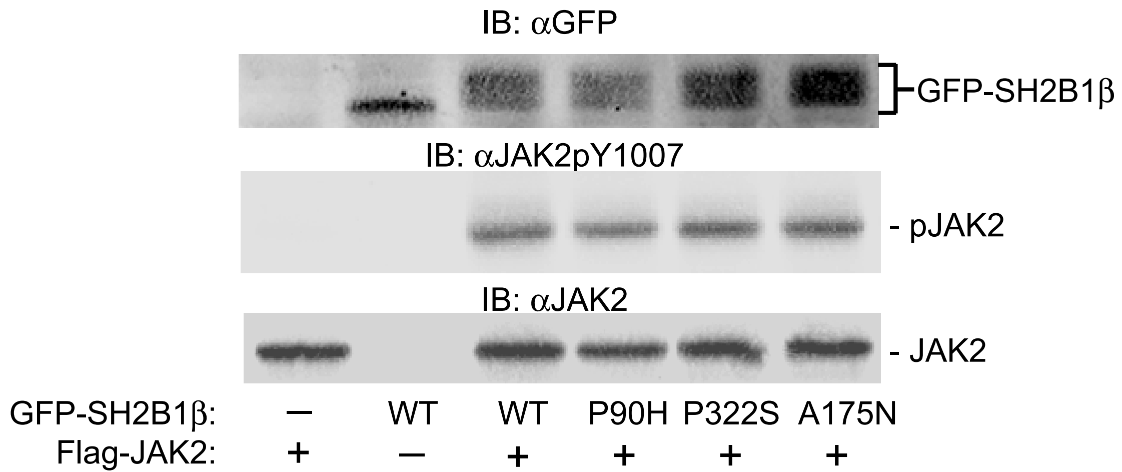


FIG. 2.6. SH2B1β P90H, P322S and A175N express at the appropriate size and to a similar extent as WT. They also exhibit similar enhancement of JAK2 phosphorylation. Proteins in lysates from 293T cells transiently expressing Flag-JAK2 and GFP-SH2B1β WT, P90H, P322S, or A175N (as indicated) were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-JAK2pY1007 (middle panel), or α-JAK2 (bottom panel).

expression and was unable to activate JAK2 (FIG. 2.7), presumably due to the fact that it lacks the SH2 domain previously shown to be required for JAK2 activation (22).

SH2B1β F344LfsX20 was also present at lower levels in the plasma membrane relative to the cytoplasm in both 293T and PC12 cells and was found in aggregates in the cytoplasm (FIG. 2.8 and FIG. 2.9). SH2B1β P90H, A175N, and P322S exhibited a steady-state subcellular distribution similar to that of WT SH2B1β in 293T and PC12 cells (FIG. 2.8 and FIG. 2.9).

We assessed the ability of the SH2B1β mutants to enhance leptin signaling by examining their ability to stimulate leptin-dependent tyrosyl phosphorylation of IRS2. Consistent with the findings on JAK2 activation, the SH2B1β point mutants were as effective as WT SH2B1β in stimulating leptin-dependent tyrosine phosphorylation of IRS2 (FIG. 2.10); similar results were seen for insulin-stimulated tyrosine phosphorylation of IRS2 (FIG. 2.11). As these latter assays rely on the overexpression of both SH2B1β and IRS2, it is possible that any subtle effects of the SH2B1 mutants were masked.

We also postulated that some of these heterozygous mutations may affect the ability of SH2B1 to dimerize. GFP-tagged WT SH2B1β co-immunoprecipitated with Flag-tagged WT SH2B1β, while the mutant 3AD/2FA (in which the phenylalanine zipper that is required for dimerization is mutated) exhibited greatly diminished coimmunoprecipitation (FIG. 2.12). Binding of the GFP-tagged P90H and A175N mutants to Flag-tagged WT SH2B1β was similar to that seen with GFP-tagged WT; however, the P322S mutation enhanced

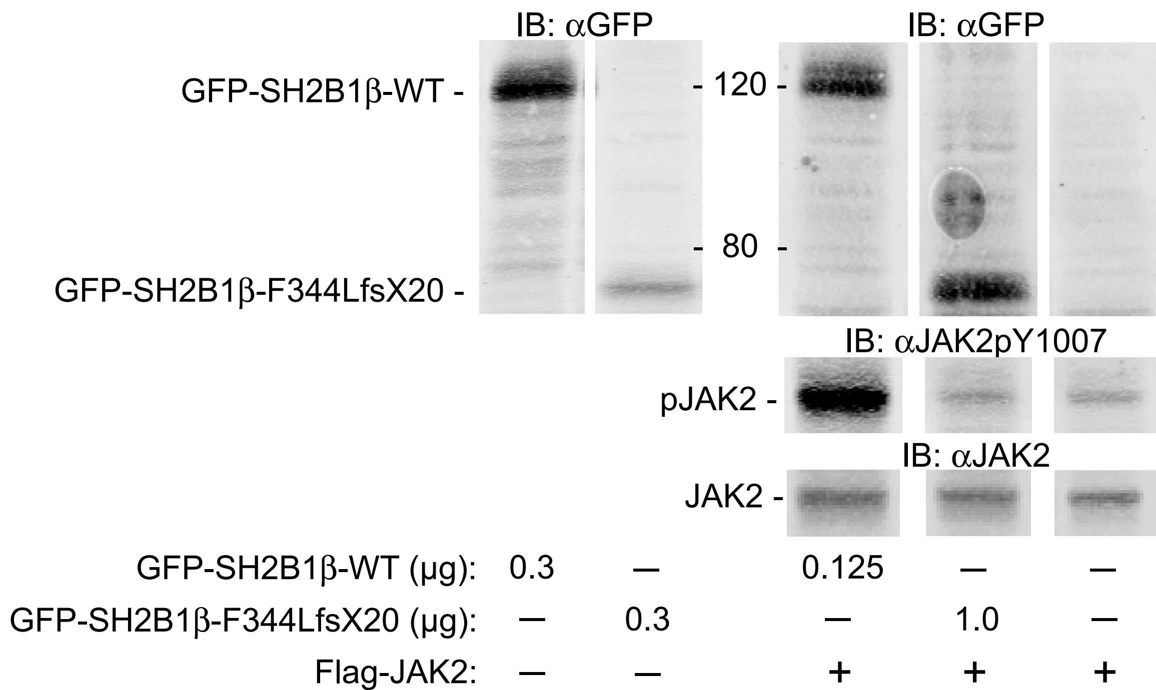


FIG. 2.7. SH2B1β truncation mutant F344LfsX20 expresses at the appropriate size but at a much lower level than WT. It does not enhance JAK2 phosphorylation. Proteins in lysates from 293T cells transiently expressing Flag-JAK2 (0.5 μg cDNA) and GFP-SH2B1β WT or F344LfsX20 (at the indicated μg cDNA amount) were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-JAK2pY1007 (middle panel), or α-JAK2 (bottom panel). The migrations of molecular weight standards are indicated in the top panel.

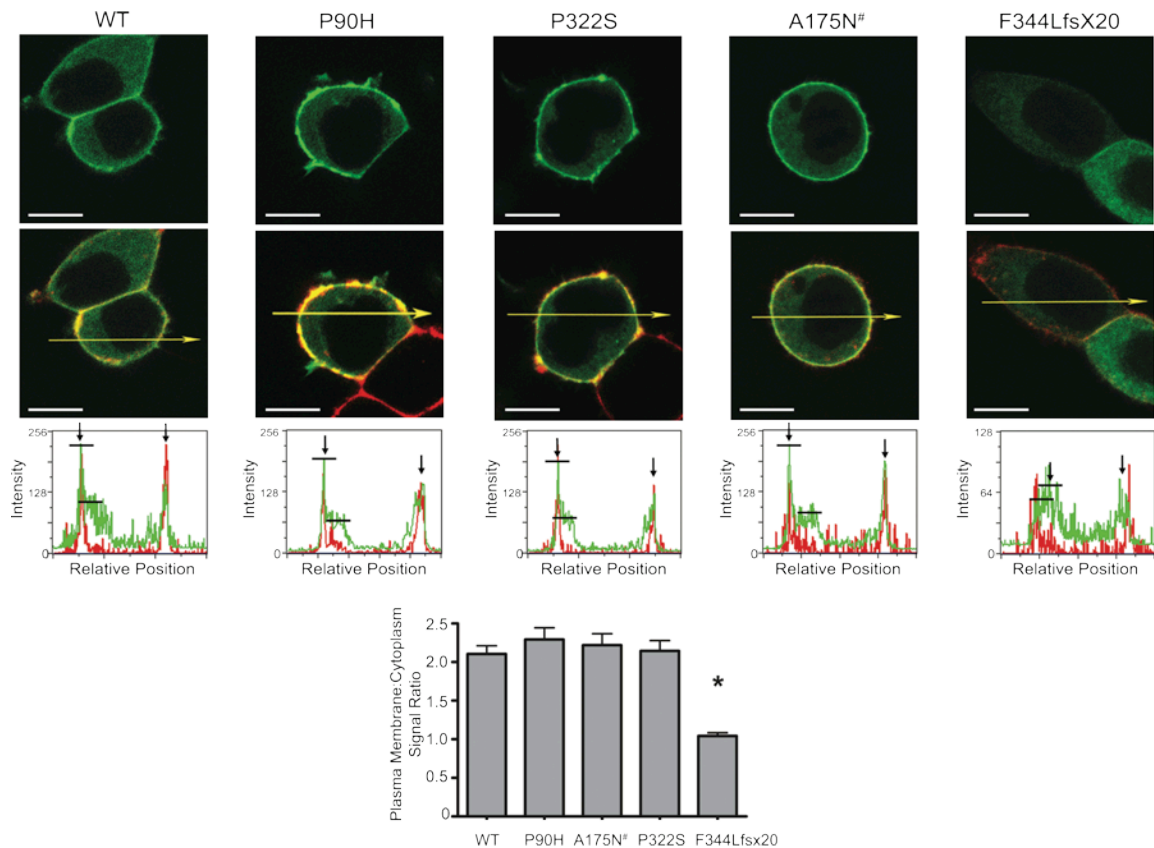


FIG. 2.8. Subcellular distribution of SH2B1β WT and mutant proteins in 293T cells. Live 293T cells transiently expressing GFP-tagged WT, P90H, A175N, P322S, or F344LfsX20 SH2B1β were stained with the plasma membrane marker wheat germ agglutinin Alexa Fluor 594 (red) and imaged using confocal fluorescence microscopy. Shown are GFP fluorescence alone (green; top) and overlay of GFP and plasma membrane marker (bottom). Scale bars: 10 μm. Below, green and red signal intensity along the yellow arrows was determined using MetaVue Linescan. Arrows (red signal intensity peak) indicate position of the plasma membrane on the linescan. Horizontal lines on the linescan graphs denote the plasma membrane and cytoplasm values used to determine the plasma membrane/cytoplasm green signal intensity ratios (means ± SEM). The SH2B1β truncation mutant localized to the PM to a lesser extent than did SH2B1β WT. *$P < 0.0001$ vs. WT.

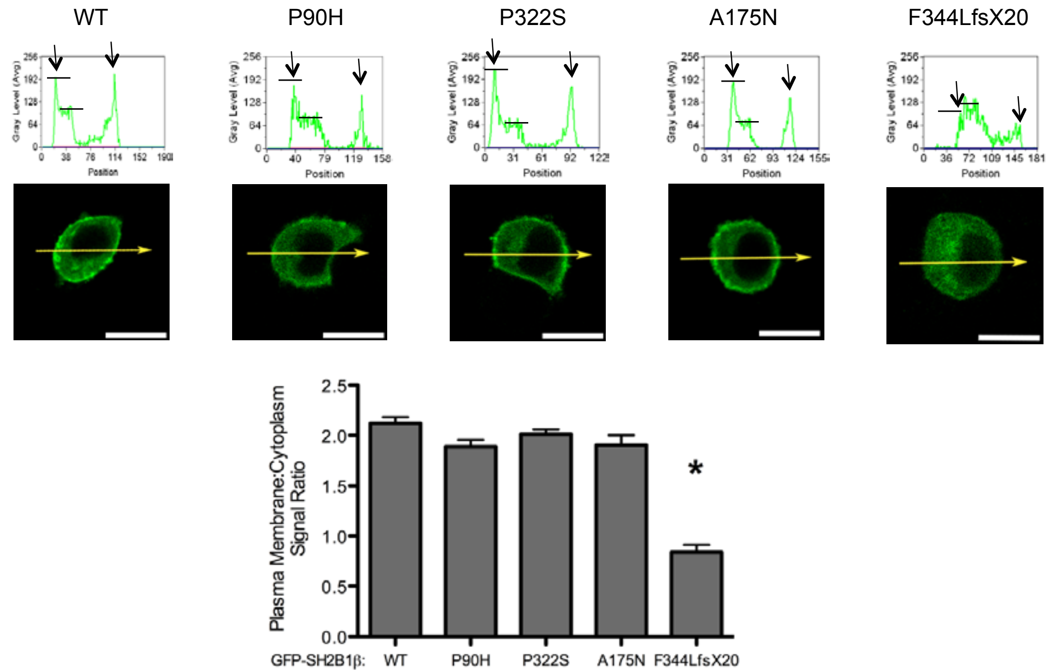


FIG. 2.9. Subcellular distribution of SH2B1β WT and mutant proteins in PC12 cells. Live PC12 cells transiently expressing GFP-tagged WT, P90H, A175N, P322S, or F344LfsX20 SH2B1β were imaged using confocal fluorescence microscopy. Scale bars: 10 μm. Green signal intensity along the yellow arrows was determined using MetaVue Linescan. Arrows indicate position of the plasma membrane on the linescan. Horizontal lines on the linescan graphs denote the plasma membrane and cytoplasm values used to determine the plasma membrane/cytoplasm green signal intensity ratios (means ± SEM). The SH2B1β truncation mutant localized to the PM to a lesser extent than did SH2B1β WT. *P < 0.0001 vs. WT.

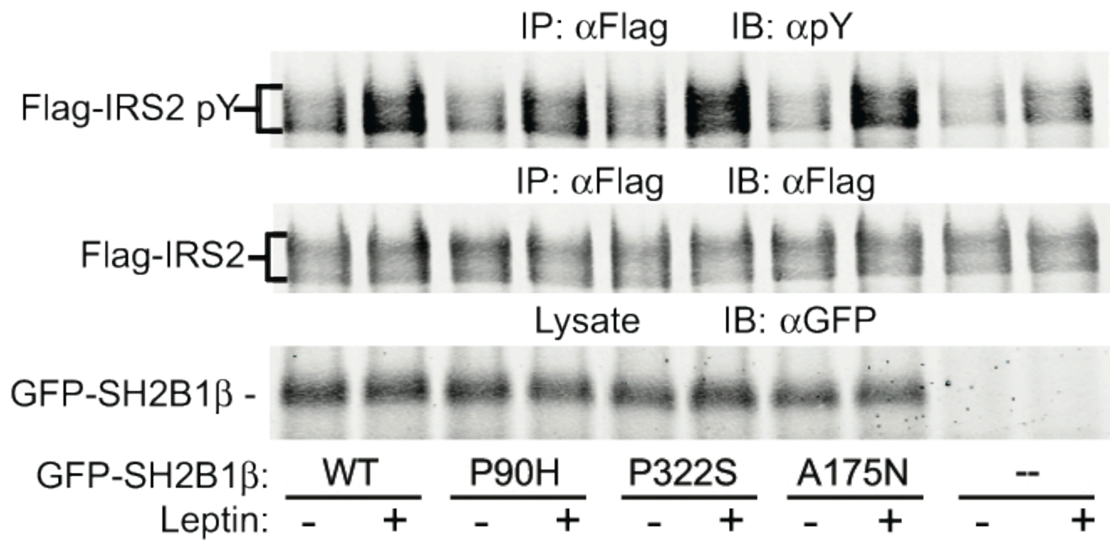


FIG. 2.10. SH2B1β mutants enhance leptin-induced tyrosyl phosphorylation of IRS2 to the same extent as SH2B1β WT. Flag-IRS2 and GFP-SH2B1β WT, P90H, P322S or A175N were transiently co-expressed (as indicated) in HEK293 cells stably expressing leptin receptor and treated with leptin (100 ng/ml) for 10 minutes. Flag-IRS2 was immunoprecipitated with α-Flag and immunoblotted with α-phospho-tyrosine 4G10 (top panel) or α-Flag (middle panel). Total lysates were blotted with α-GFP (bottom panel).

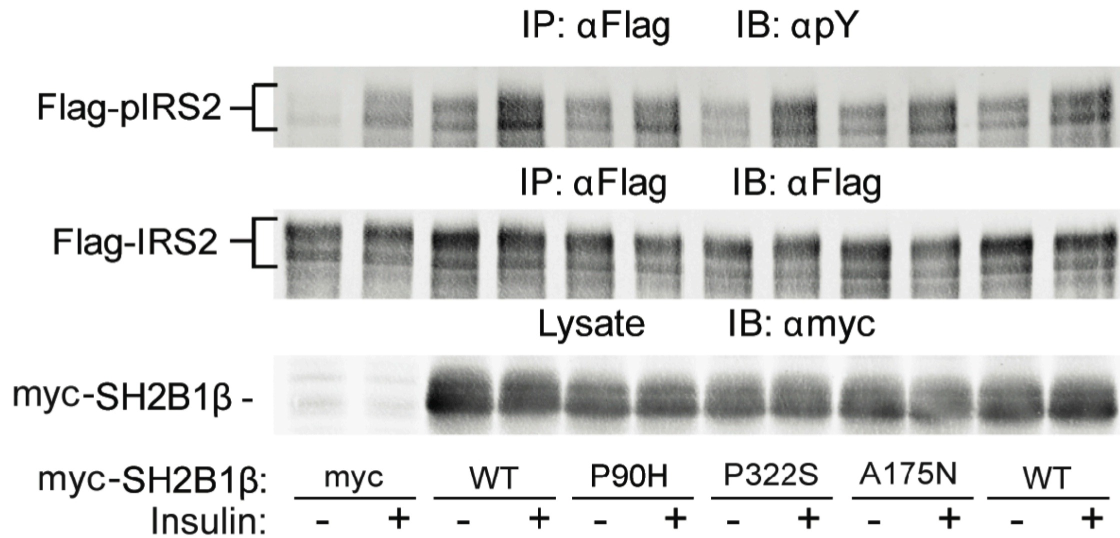


FIG. 2.11. SH2B1β mutants enhance insulin-induced tyrosyl phosphorylation of IRS2 to the same extent as SH2B1β WT. Flag-IRS2 and GFP-SH2B1β WT, P90H, P322S or A175N were transiently co-expressed (as indicated) in 293T cells and treated with 2.5 nM insulin for 30 minutes. Flag-IRS2 was immunoprecipitated with α-Flag and immunoblotted with α-phosphotyrosine 4G10 (top panel) or α-Flag (middle panel). Total lysates were blotted with α-myc (bottom panel).

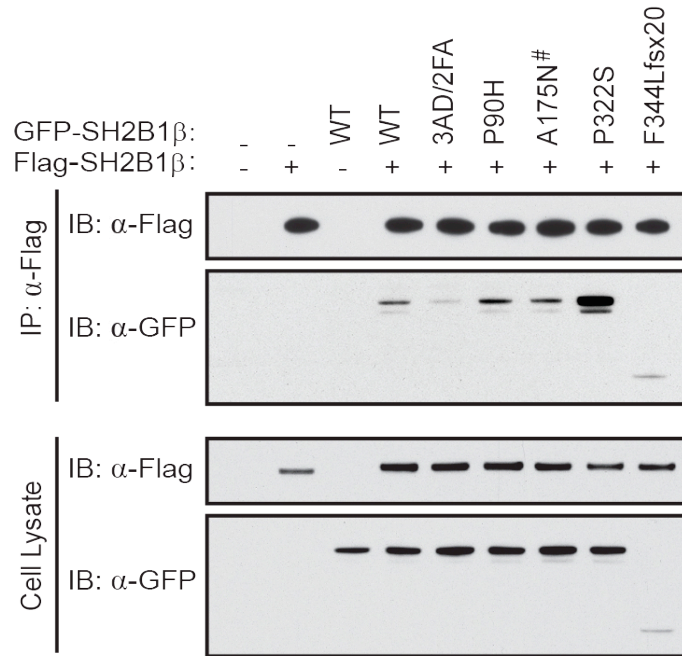


FIG. 2.12. Dimerization of SH2B1β with human mutations. Flag-tagged SH2B1β was immunoprecipitated from HEK293 cells coexpressing Flag- tagged WT SH2B1β and GFP-tagged SH2B1β mutants (including the dimerization mutant 3AD/2FA). Immunoprecipitated proteins and proteins in cell lysates were immunoblotted using anti-Flag and anti-GFP antibodies. Binding of the P90H and A175N mutants was similar to that seen with WT; however, the P322S mutation enhanced dimerization. Note that for the A175N mutant, all experiments were performed using rat SH2B1β (NP_001041645), in which Thr175 is Ala175.

dimerization.

We have demonstrated that loss-of-function mutations in SH2B1 were associated with severe early-onset obesity, insulin resistance, and reduced final height. All the mutations were associated with loss of function in assays of GH/NGF-mediated signaling. Intriguingly, apart from the frameshift mutation, the other mutants did not impair leptin signaling. Although this discordance may reflect the differing sensitivities of the assays used, it is plausible that some of the effects of SH2B1 on energy homeostasis may be mediated by leptin-independent pathways.

Unexpectedly, we observed that mutations were associated with a range of behavioral abnormalities, including a tendency for social isolation and high levels of aggression, as reported by healthcare professionals and family members. We recognize that the absence of formal psychiatric testing and disease classification in these individuals and in the controls is a limitation of our study. However, we note that these phenotypes were not seen in association with the other genetic obesity syndromes we have characterized to date (106, 107), although the socioeconomic status of mutation carriers was comparable. As we observed impaired NGF-induced neuronal differentiation *in vitro*, a similar response to other ligands, such as centrally expressed neurotrophins, could contribute to these features (14). The maladaptive behaviors reported in mutation carriers compared with controls were not reported in previous studies in mice. Our studies imply an unexpected role for SH2B1 in human behavior.

Methods

SH2B1 mutation analysis. All 300 patients from the GOOS study that fit the criteria for obesity and severe insulin resistance were screened for mutations in the *SH2B1* gene. An a priori power calculation was not conducted; given the rarity of these phenotypes, all eligible patients available to us were studied. Primers were designed to cover the coding sequence (NM_015503) and splice junctions of *SH2B1*. Mutation screening was undertaken by PCR, followed by direct sequencing using BigDye terminator chemistry (Applied Biosystems) and analysis on an ABI 3730 automated sequencer (Applied Biosystems). 500 controls were also sequenced using the same methods.

SH2B1 gene expression in human tissues. A human tissue cDNA library was prepared using 1 μl RNA (Clontech), which was reverse transcribed to cDNA using a RetroScript kit (Ambion). 1 μl cDNA was used as a template in an RT-PCR reaction (forward primer, 5′-CAGCTATGTCCCATCCTCCCAGCGA-3′; reverse primer, 5′-CAGGCTGTGGGGGATCTGTCCATGAAG-3′). The four SH2B1 isoforms were amplified simultaneously, generating four PCR products of distinct size (SH2B1α, 102 bp; SH2B1β, 201 bp; SH2B1γ, 254 bp; SH2B1δ, 154 bp).

Expression plasmids. The PC12 and RAW264.7 cells used to

characterize the functional impact of the mutants are derived from rat and mouse respectively, so we used a cDNA encoding a rodent (rat) SH2B1β. Mutant GFP-SH2B1β cDNA was created using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mouse IRS2 was cloned into pcDNA(3.1-) vector (Flag-tag at N-terminus).

Cell culture and transfection. Q293A cells stably expressing the long form of the mouse leptin receptor (293^{LRb})(110), HEK 293 cells, and 293T cells were transiently transfected using the polyethylenimine (PEI) method. PC12 cells were cultured in RPMI 1640 (ATCC) containing 10% horse serum (Invitrogen) and 5% fetal bovine serum (FBS) (Atlanta Biologicals) on collagen-coated dishes and transfected as described previously (35). RAW264.7 cells (J. Swanson, University of Michigan) were cultured in DMEM (Invitrogen) supplemented with 8% heat inactivated FBS (Atlanta Biologicals), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen) at 37°C in 5% CO_2. RAW264.7 cells were transiently transfected using Amaxa nucleofector (Lonza) using solution V and setting D32.

Neuronal differentiation of PC12 cells. Transfected PC12 cells were plated on collagen-coated 60-mm dishes. 24 hours post transfection, 25 or 50 ng/ml NGF (BD Bioscience) was added to RPMI 1640 containing 5% horse serum, 1% FBS to induce PC12 cell differentiation. NGF-containing differentiation medium was refreshed two days later. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200). The percent of GFP+ cells that were differentiated (neurite outgrowths >2 times the length of the cell body)

was determined.

Cell Imaging. Transfected 293T cells plated on 35-mm, poly-d-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA) were incubated for 8 hours in growth medium supplemented with 20 nM leptomycin B (Sigma) to inhibit Crm1-dependent nuclear export. GFP+ cells were visualized with an Olympus FluoView 500 laser scanning confocal microscope using a 60x water-immersion objective and FluoView version 5.0 software. Linescan profiles were obtained and analyzed using MetaVue Software (Universal Imaging, Sunnyvale, CA).

GH-induced migration of RAW264.7 macrophages. RAW264.7 cells transiently expressing SH2B1β WT or mutant were incubated in growth medium for 24h, then in serum-free medium (1% BSA) overnight. Transfected cells suspended in serum-free medium were added to the upper chamber of a Transwell (Costar) unit with 5-μm pore size; the lower chamber contained serum-free medium with 500 ng/ml recombinant human GH (gift of Eli Lilly & Co.). Transwell units were incubated at 37°C for 18 h, fixed in methanol and air-dried. Cells in the upper chamber were removed with a cotton swab. Filter membranes were stained with hematoxylin and eosin (H&E) (1:10 dilution) for 1 hour and then washed with ddH2O. Cells that had migrated were counted (≥3 independent fields per condition) under a light microscope.

SH2B1 dimerization. HEK293 cells were transiently transfected with Flag- and GFP-tagged SH2B1β constructs. 36h post-transfection, cells were washed with PBS, harvested in NP-40 lysis buffer and clarified by centrifugation. Lysate

(3 mg protein) was pre-cleared by incubation with Protein A agarose (Sigma) at 4°C for 30 min before incubation with anti-FLAG M2 affinity gel (Sigma) at 4°C overnight. Immunoprecipitates were washed, resuspended in sample buffer and filtered through a Spin-X filter to remove the resin. NuPAGE reducing agent (1X; Invitrogen) was added to the eluted samples, which were subjected to electrophoresis and immunoblot analysis.

Statistics. To test for statistically significant differences in values from the neuronal differentiation assay (n=4-8 experiments, 60-120 cells/condition/experiment), a one-way ANOVA with a Dunnett's multiple comparison post-test was applied. For the values from each day of the assay (days 1-4), the ANOVA yielded *p*-values < 0.0001 and the Dunnett's post-test showed significant differences between GFP-SH2B1β WT-expressing cells and every other condition (alpha = 0.05). For nuclear accumulation of GFP-SH2B1β (n=3-5 experiments, 76-150 cells/condition/experiment) and subcellular localization (n=16-26), statistical significance was determined using an unpaired, two-tailed Student's *t*-test. For the motility assay (n=4), a paired, one-tailed Student's *t*-test was used. 2-sided Fisher's exact test was used to compare prevalence of mutations in cases versus controls. A P-value less than 0.05 was considered significant.

Study subjects. The Genetics of Obesity Study (GOOS) cohort consists of 4300 probands with severe obesity (Body Mass Index Standard Deviation Score (BMI SDS) >3) before 10 years of age. BMI SDS values were calculated using UK reference data (111). We identified three hundred patients who also

exhibited disproportionate insulin resistance as defined by the presence of acanthosis nigricans, the development of type 2 diabetes in early adolescence and/or markedly elevated plasma insulin levels (top decile for age, gender and body mass index). The mean (±SD) BMI SDS for this group of 300 chosen for study was 4.3 ± 1.4. In adults, overweight was defined as BMI 25-29.9 kg/m^2, obesity as BMI > 30 kg/m^2 according to WHO criteria. In children (<18yrs), we used definitions proposed by the International Obesity Task Force: overweight defined as >91st and obesity as >99th percentile for age-adjusted BMI.

Chapter 3

Effects of human mutations on cellular actions of SH2B1 alpha

Abstract

SH2B1 modulates signaling by a variety of ligands that bind to receptor tyrosine kinases or JAK-associated cytokine receptors, including leptin, insulin, growth hormone (GH), and nerve growth factor (NGF). Targeted deletion of *Sh2b1* in mice results in increased food intake, obesity, and insulin resistance, with an intermediate phenotype seen in heterozygous null mice on a high-fat diet. SH2B1 is expressed as four different isoforms alpha, beta, gamma, and delta. Previously, *SH2B1* loss-of-function mutations have been identified in a large cohort of patients with severe early-onset obesity and the effects of which on cellular actions of SH2B1β were investigated. Here, we identify additional mutations present in *SH2B1* from obese patients with disproportionate insulin resistance, including three mutations exclusive to the alpha isoform. We investigated the effects of these mutations on cellular actions of SH2B1α. We did not see a difference between SH2B1α WT or mutant to enhance JAK2 activation, promote insulin-mediated IRS2 phosphorylation, or localize at the plasma membrane. In contrast to SH2B1β, SH2B1α does not enhance NGF-induced neurite outgrowth or uPAR expression and does not cycle through the

nucleus. We show that it is mostly the unique C-terminal tail of SH2B1α that does not allow it to enhance NGF-induced neurite outgrowth.

Introduction

Recently, *SH2B1* has been identified as an obesity gene. Humans with point mutations found in *SH2B1* exhibit severe early-onset obesity, extreme insulin resistance, and maladaptive behavior (112). The effects of these mutations on the cellular actions of SH2B1 were investigated with the beta isoform of SH2B1 (112). SH2B1β with human mutations showed impaired enhancement of NGF-induced neuronal differentiation, nuclear accumulation, and growth hormone-induced macrophage motility. The hyperphagia and maladaptive behavior seen in these patients hint at a possible role for SH2B1 in neuronal development. This is supported by the effects of neuronal SH2B1 in mice with a whole-body knockout of *Sh2b1*. *Sh2b1*$^{-/-}$ mice are obese and insulin resistant (18), yet when transgenic mice are created using a neuronal-specific enolase promoter to express SH2B1β only neurons, the metabolic disorder phenotype is ameliorated (54). In addition to mutations P90H, T175N, P322S, and F344LfsX20 that were first reported (112), more mutations have been identified in *SH2B1*, including ones specific to the alpha isoform of SH2B1.

The adaptor protein SH2B1 is expressed as four different isoforms (alpha, beta, gamma and delta) that arise due to alternative splicing (28, 29). The SH2B1 isoforms are identical in their sequence until the variable splice site

produces unique C-terminal tails for each. While the beta isoform has been well characterized, there has not been much investigation into the alpha isoform.

The alpha isoform has been seen to behave similarly to SH2B1β in promoting mitogenesis and cell survival in NIH 3T3 fibroblasts (28), and insulin receptor kinase activity in 3T3-L1 adipocytes (49). SH2B1β and γ have been reported to enhance NGF-induced PC12 cell neuronal differentiation (30, 31), in part by promoting the expression of a sub-set of NGF-induced genes required for differentiation (67). Nuclear-cytoplasmic shuttling of SH2B1β appears to be required for this enhancement of NGF-induced neuronal differentiation (62). Human mutations identified in *SH2B1* impair the ability of SH2B1β to enhance NGF-induced neuronal differentiation (112).

An N-terminal nuclear localization signal (NLS) and nuclear export sequence (NES) allow for the nuclear shuttling of SH2B1β. The NLS and NES motifs present in SH2B1β are also present in SH2B1α, suggesting SH2B1α may also cycle through the nucleus. While SH2B1β is ubiquitously expressed, SH2B1α appears to only be expressed in brain tissues (112). Thus, it is important to gain a better understanding of the cellular functions of SH2B1α, particularly in neuronal differentiation, and how these are affected by the human mutations.

In this report we characterize the effects of SH2B1α WT or with a human mutation incorporated on NGF-induced neuronal differentiation and gene expression, JAK2 autophosphorylation, insulin-stimulated IRS2 phosphorylation, and sub-cellular localization of SH2B1.

Methods and Materials

Antibodies and reagents. αFLAG (M2) (1:2000) was from Sigma. αJAK2 p1007/1008 (1:1000) was from Upstate Biotechnology Inc. (Lake Placid, NY). αphospho-tyrosine 4G10 (1:1000) was from EMD Millipore Corporation (Billerica, MA). IRDye800-conjugated anti-GFP, IRDye700 and IRDye800 conjugated anti-mouse and anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) were used at a dilution of 1:20,000. NGF and rat-tail collagen I were purchased from BD Bioscience (San Diego, CA).

Plasmids. Human *SH2B1α* (from S. Shoelson, Joslin Diabetes Center, Boston, MA) (NM_001145795.1) and rat *SH2B1β* (NM_001048180) were cloned into the pEGFP C1 vector (Clontech, Mountain View, CA) (described previously (62)). Mutations were introduced using the QuickChange Mutagenesis kit (Stratagene). All constructs were verified by sequencing.

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 5% CO_2 in normal growth medium containing RPMI 1640 (ATCC), 10% heat-inactivated horse serum (Invitrogen), and 5% fetal bovine serum (Atlanta Biologicals). PC12 cells were transfected using a Gene

Pulser Xcell Electroporator (400V, 500μF) in a 0.4 cm cuvette (Bio-Rad). 293T cells (from Dr. O. A. MacDougald, University of Michigan, Ann Arbor, MI) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 8% calf serum (Atlanta Biologicals) and transfected using calcium phosphate.

Confocal Microscopy. Cell imaging was performed using an Olympus FluoView 500 laser scanning confocal microscope and FluoView version 5.0 software. Transfected 293T cells were plated on poly-d-lysine coated glass bottom dishes (MatTek, Ashland, MA) and imaged while in Ringer's buffer (10 mM HEPES, 155 mM NaCl, 2 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 10 mM NaH2PO4 and 10 mM glucose, pH 7.2). Linescan profiles were obtained and analyzed using MetaVue Software (Universal Imaging, Sunnyvale, CA) and has been described previously (65).

Differentiation of PC12 cells. PC12 cells were grown on collagen-coated 10-cm^2 dishes then plated on collagen-coated 60-mm^2 dishes after transfection. 24 h post transfection, 25 ng/ml NGF was added to RPMI 1640 containing 5% horse serum, 1% FBS to induce PC12 cell differentiation. NGF-containing differentiation medium was refreshed two days later. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200). The percent of GFP+ cells that were differentiated (neurite outgrowths >2 times the length of the cell body) was determined.

Cell lysis and immunoblotting. Cells were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample lysis buffer (50 mM Tris [pH 7.5], 1% SDS, 0.001% bromophenol blue, 10% glycerol, 0.1% Triton

X-100, 150 mM NaCl, 2 mM EGTA) and proteins were separated by SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with the indicated antibodies. Blots were then incubated with infrared dye-conjugated secondary antibodies and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

qPCR. Total RNA was isolated using Stat60 (Tel-Test) and cDNA was generated using TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). Gene expression was assessed using SYBR Green (Bio-Rad) and the Eppendorf Mastercycler ep (Hamburg, Germany). All readings were normalized to the expression of glyceraldehyde-3- phosphate dehydrogenase (GAPDH), which did not differ between the different transfected cells.

Results

Additional human mutations identified in *SH2B1*. The human mutations identified previously to affect the function of SH2B1β are also present in SH2B1α. Since the publication of the initial report characterizing human mutations in *SH2B1* (112), more mutations in *SH2B1* from patients with early onset obesity and extreme insulin resistance have been identified. Most mutations are common to all SH2B1 isoforms, including the previously reported P90H, T175N, P322S, F344LfsX20, and the novel G238C, R270W, A484T and T546A. However, some mutations have been identified that are present in only the SH2B1 alpha (or delta) isoform; these include: A663V (which corresponds to a R680C in the delta isoform), V695M (which does not result in an altered amino acid sequence in the delta isoform), and A723V. (FIG. 3.1) The effects of these mutations on SH2B1α cellular function are characterized in this report.

SH2B1α enhances JAK2 autophosphorylation. SH2B1β was originally identified as a binding partner for the receptor-associated tyrosine kinase JAK2 (22). Many cytokine and growth factor receptors utilize JAK2 to mediate their signal transduction, including the receptors growth hormone (GH) and leptin. When co-expressed with JAK2, SH2B1β has been shown to enhance

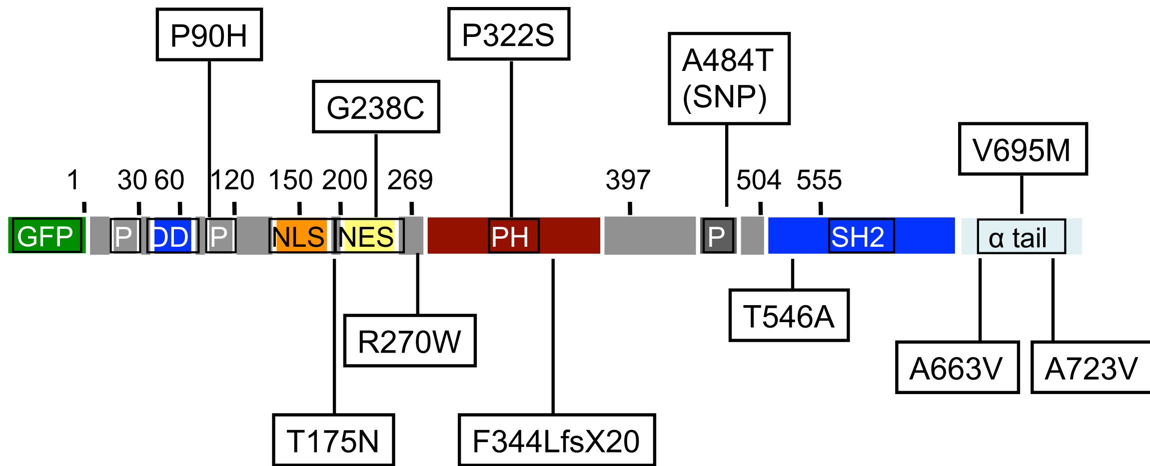


FIG. 3.1. Identification of human mutations in SH2B1α. P, proline-rich region; DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain, SH2B1α unique C-terminal tail in light blue. SH2B1α mutations are shown in boxes.

JAK2 tyrosine kinase signaling activity. This requires an intact SH2 domain of SH2B1β to bind to phospho-tyrosine 813 of JAK2 (40, 113).

To determine whether SH2B1α, like SH2B1β, activates JAK2, 293T cells were transiently transfected with Flag-tagged JAK2 with or without GFP-tagged SH2B1α WT. JAK2 activity was assessed by blotting with α-pJAK2 Y1007/1008, which recognizes the active form of JAK2. SH2B1α WT is able to enhance JAK2 activity, similarly to what is seen when JAK2 is co-expressed with SH2B1β (FIG. 3.2). Next we tested whether human mutations identified in *SH2B1* impaired the ability of SH2B1α to activate JAK2. SH2B1α with human mutations P90H, T175N, G238C, R270W, P322S, A484T, T546A, A663V, V695M, and A723V all express at the expected size and intensity. FIG. 3.2 shows that 10 of the mutants enhance JAK2 activity to the same extent as SH2B1α WT. However, SH2B1α F344LfsX20 expressed at a much lower intensity than SH2B1α WT and was unable to promote the autophosphorylation of JAK2, consistent with what was seen with SH2B1β F344LfsX20 (see FIG. 2.7).

SH2B1α F344LfsX20 and SH2B1β F344LfsX20 are expected to encode the same protein product since the mutation produces a truncated protein consisting of only the shared region of SH2B1 common to all four isoforms. The SH2B1α F344LfsX20 truncated protein product does not contain the SH2 domain of SH2B1, which is responsible for binding to phospho-tyrosine residues and mediating tyrosine kinase signaling through SH2B1. Thus, it was not surprising that it was unable to activate JAK2.

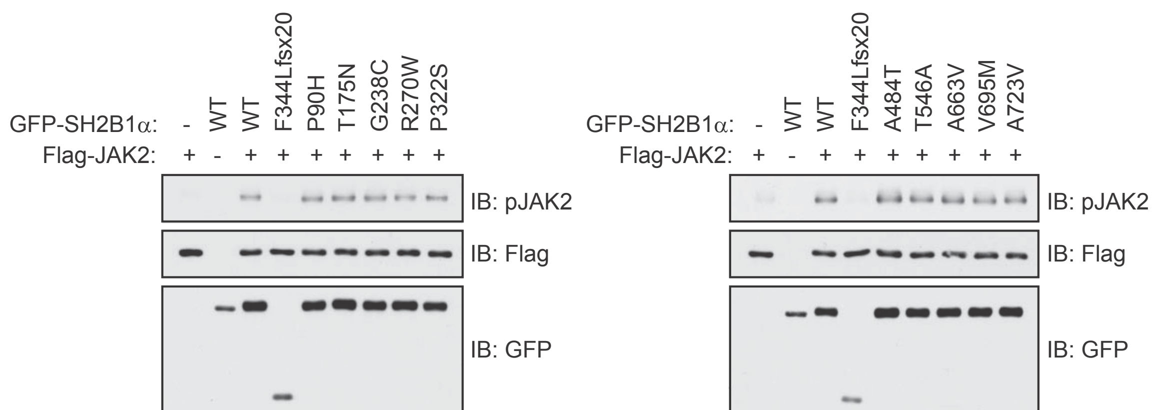


FIG. 3.2. SH2B1α point mutants but not F344LfsX20 enhance JAK2 activity. Proteins in lysates from 293T cells transiently expressing Flag-JAK2 and GFP-SH2B1α WT, F344LfsX20, P90H, P322S, T175N, G238C, R270W, P322S, A484T, T546A, A663V, V695M, or A723V (as indicated) were separated by SDS-PAGE and immunoblotted with α-JAK2pY1007 (top panel), α-Flag (middle panel), or α-GFP (bottom panel).

SH2B1α point mutants promote insulin-mediated IRS2 phosphorylation. SH2B1β has been shown previously to alter insulin signaling (49, 51). Insulin signaling was dramatically impaired in muscle, liver, and adipose tissue when *Sh2b1* was knocked out, whereas SH2B1β overexpression enhanced insulin receptor autophosphorylation and tyrosine phosphorylation of insulin receptor substrates (IRS) 1 and 2 (51). To assess the effect of SH2B1α with human mutations on insulin signaling, 293T cells were transiently transfected with Flag-tagged IRS2 and either GFP-tagged SH2B1α WT or SH2B1α with a human mutation. SH2B1α WT is able to enhance the insulin-stimulated tyrosyl phosphorylation of IRS2, similar to SH2B1β (FIG. 3.3). The SH2B1α point mutants P90H, T175N, G238C, R270W, and P322S enhance insulin-stimulated tyrosyl phosphorylation of IRS2 to the same extent as SH2B1α WT. Consistent with what was seen from the JAK2 signaling assay, SH2B1α F344LfsX20 failed to promote the tyrosyl phosphorylation of IRS2 in response to insulin (FIG. 3.3). It was not expected that SH2B1α F344LfsX20 would enhance insulin signaling because the SH2 domain of SH2B1 is required for SH2B1 to bind phospho-tyrosines in the active insulin receptor as well.

SH2B1α does not enhance NGF-induced neuronal differentiation or uPAR expression. To assess whether SH2B1α, like SH2B1β, is able to enhance NGF-induced neuronal differentiation, PC12 cells were transiently transfected with GFP-SH2B1β or GFP-SH2B1α. Overexpression of SH2B1β greatly enhanced NGF-induced PC12 cell neuronal differentiation over GFP-

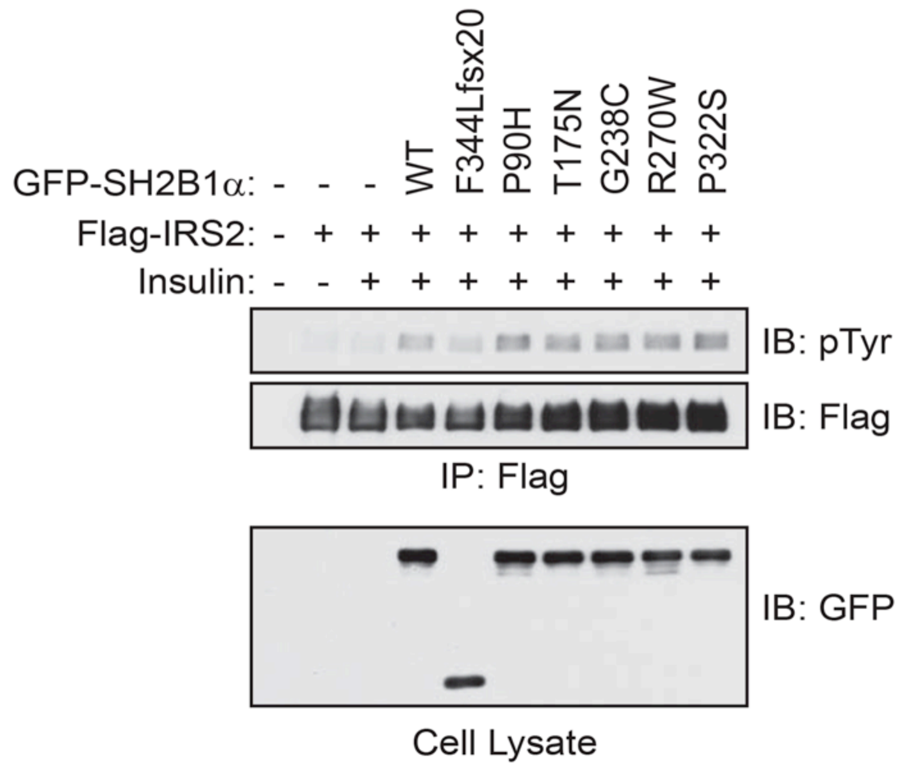


FIG. 3.3. SH2B1α point mutants but not F344LfsX20 enhance insulin-induced IRS2 tyrosyl phosphorylation. GFP-tagged human SH2B1α mutants were expressed along with Flag-tagged IRS2 in HEK293 cells. Transfected cells were serum-starved for 16hrs and then treated with 100nM insulin for 5min. Cell lysates were subjected to immunoprecipitation with Flag-agarose beads and immunoblotting was carried out using the indicated antibodies.

expressing control cells as seen previously (112). In contrast, overexpression of SH2B1α had no stimulatory effect (FIG. 3.4).

NGF-induced expression of uPAR is required for neuronal differentiation (69) and has been seen to depend on SH2B1 for its full NGF-induced expression. When SH2B1 expression is decreased using shRNA, NGF-induced expression of uPAR is decreased. Conversely, NGF-induced expression of uPAR is increased when SH2B1β is overexpressed in PC12 cells (62, 67). We therefore looked to see if SH2B1α enhanced NGF-induced expression of uPAR. In PC12 cells stably expressing GFP-SH2B1α and stimulated with NGF for 6 hours, uPAR expression was not significantly higher than in control cells. In contrast, PC12 cells transfected with SH2B1δ, which we have shown to enhance NGF-induced neuronal differentiation (described in chapter 4), promoted a large enhancement of NGF-induced uPAR expression (FIG. 3.5).

SH2B1α does not cycle through the nucleus. Previous work suggests that nuclear entry is critical for SH2B1 to promote NGF-induced differentiation and gene expression (62). To assess whether SH2B1α is capable of shuttling through the nucleus as SH2B1β is, 293T cells were transiently transfected with GFP-SH2B1β or GFP-SH2B1α and incubated with the nuclear export inhibitor leptomycin B (LMB) for 13 hours. Under steady-state conditions, SH2B1α and SH2B1β both appear localized at the plasma membrane (PM) and cytosol. However, in contrast to SH2B1β, which showed substantial nuclear accumulation with LMB treatment, SH2B1α failed to accumulate in the nucleus

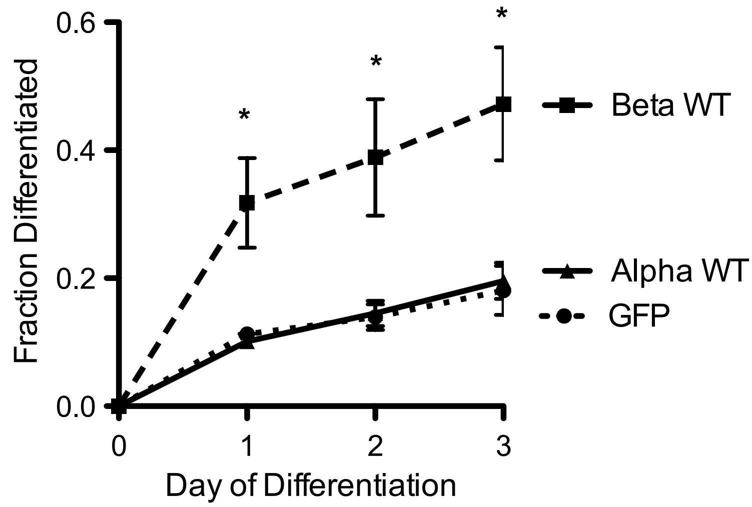


FIG. 3.4. SH2B1α does not enhance NGF-induced PC12 neurite outgrowth. PC12 cells expressing GFP, GFP-SH2B1β, or GFP-SH2B1α were treated with NGF (25 ng/ml) to induce differentiation. Neurites > 2X cell body length were counted as differentiated. Means ± SEM, n = 3, * p,0.05.

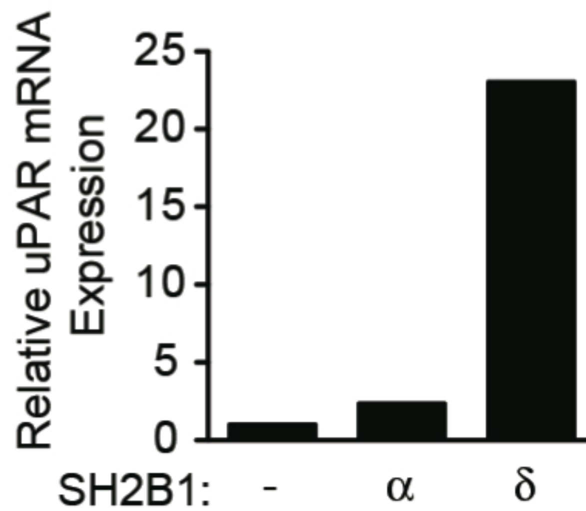


FIG. 3.5. SH2B1α does not enhance NGF-induced uPAR expression. PC12 cells expressing GFP (-), GFP- SH2B1α or GFP-SH2B1δ were treated with NGF (25 ng/ml) to induce differentiation for 6h. uPAR mRNA was analyzed by qPCR. Levels of uPAR mRNA were normalized to levels of GAPDH mRNA and then further normalized to levels of mRNA in control cells (GFP). Representative experiment.

with LMB (FIG. 3.6). This result suggests that SH2B1α does not constitutively shuttle through the nucleus as SH2B1β does, providing one possible explanation for why SH2B1α is unable to promote NGF-induced neuronal differentiation and gene expression.

SH2B1α is localized at the PM to a lesser extent than SH2B1β. Because SH2B1α does not enter the nucleus as SH2B1β does, we wanted to determine whether SH2B1α localizes differently at other sub-cellular compartments compared to SH2B1β. To assess this, 293T cells were transiently transfected with GFP-SH2B1β or GFP-SH2B1α WT and live cells were imaged by confocal microscopy. Linescans using MetaVue software were used to measure the fluorescence signal intensities for GFP-SH2B1 emanating from the plasma membrane (PM) and cytosol. While the PM:cytosol signal intensity ratio for SH2B1β is greater than 2, the PM:cytosol signal intensity ratio for SH2B1α WT is significantly less at ~1.5 (FIG. 3.7). To determine if serum levels in the medium affect the PM:cytosol ratio of SH2B1, 293T cells were transiently transfected with GFP-SH2B1β or GFP-SH2B1α and incubated with 8% calf serum or without serum for 16 hours. Live cells were imaged using confocal microscopy. Serum increased the amount of SH2B1β at the PM (PM:cytosol signal intensity ratio >2 with serum compared to ~1.5 without). In contrast, SH2B1α did not show a change in PM localization with serum (FIG. 3.8). In the absence of serum, SH2B1α and SH2B1β have similar PM:cytosol ratios. However, serum increases the amount of SH2B1β at the PM but not the amount of SH2B1α. Although SH2B1α is capable of mediating tyrosine kinase signaling

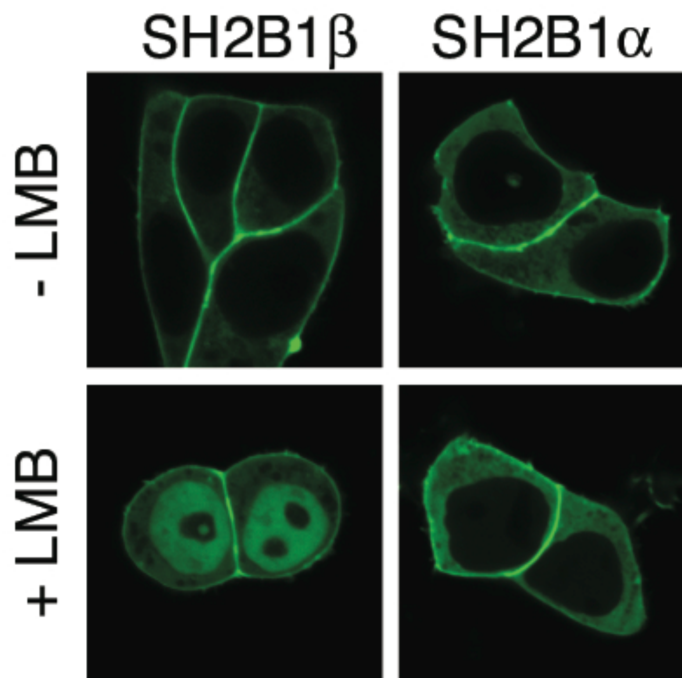


FIG. 3.6. SH2B1α exhibits impaired ability to translocate to the nucleus. Live 293T cells expressing rat GFP-SH2B1β or human GFP-SH2B1α ± LMB for 13 hours were imaged using fluorescent confocal microscopy.

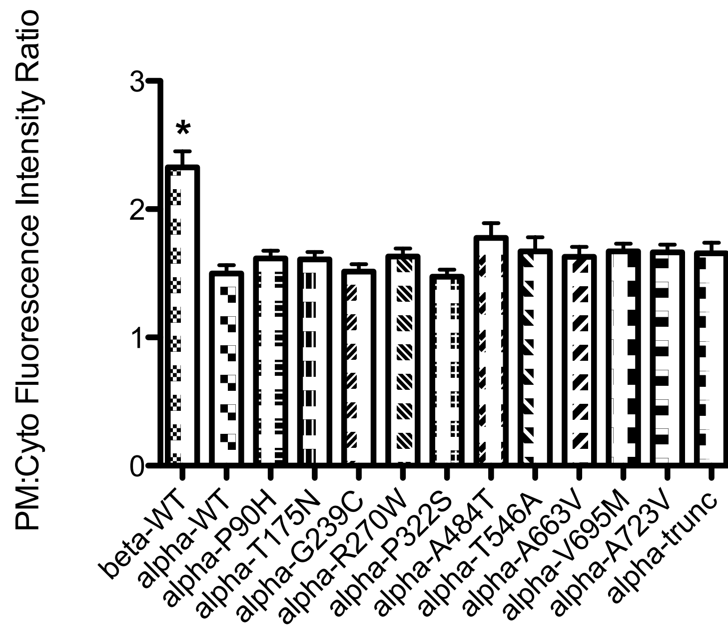


FIG. 3.7. Subcellular distribution of SH2B1α WT and mutant proteins. Live 293T cells transiently expressing GFP-tagged SH2B1β WT, SH2B1α WT, or SH2B1α with a human mutation were stained with the plasma membrane marker wheat germ agglutinin Alexa Fluor 594 and imaged using confocal fluorescence microscopy. Green and red signal intensities were determined using MetaVue Linescan. The plasma membrane:cytoplasm signal intensity ratios (PM:cyto) (means ± SEM) are shown. *P < 0.001.

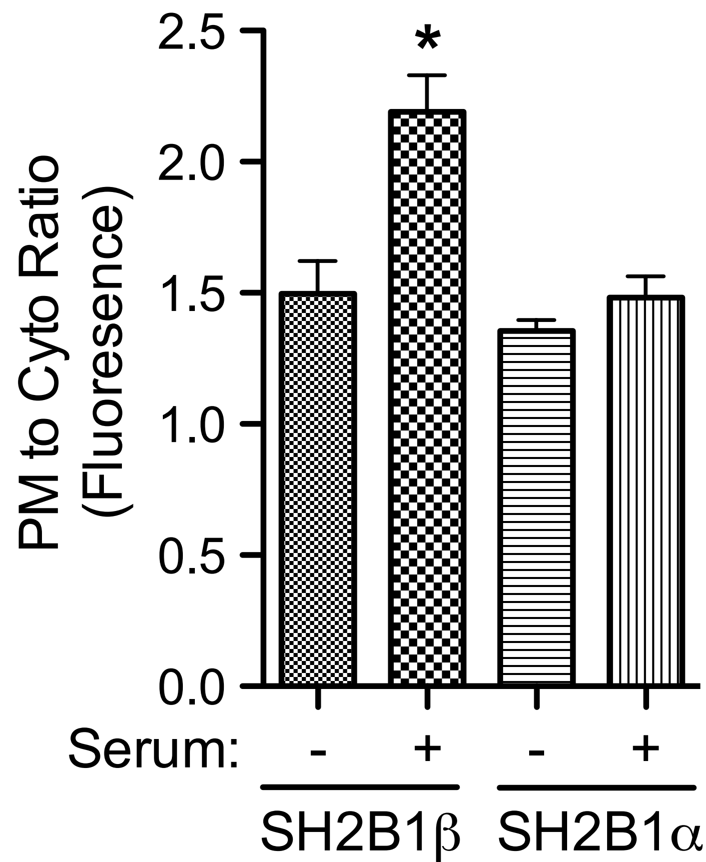


FIG. 3.8. Subcellular distribution of SH2B1β and SH2B1α WT in response to serum. Live 293T cells transiently expressing GFP-tagged SH2B1β WT or SH2B1α WT were stained with the plasma membrane marker wheat germ agglutinin Alexa Fluor 594 and imaged using confocal fluorescence microscopy. Cells without serum were deprived for ~16 h before imaging. Green and red signal intensities were determined using MetaVue Linescan. The plasma membrane:cytoplasm signal intensity ratios (means ± SEM) are shown. *P < 0.001.

(such as for the insulin receptor (49)), these data suggest that SH2B1β may be more active at the PM in response to cytokine and growth factor signaling.

The C-terminal of tail SH2B1α inhibits the ability of SH2B1 to promote NGF-induced neuronal differentiation. Because the sole differing region between the isoforms of SH2B1 is their unique C-termini, we asked whether the unique C-terminal region of SH2B1α is responsible for the inability of SH2B1α to enhance NGF-induced neuronal differentiation. To test this hypothesis, a truncated form of SH2B1 was created where a stop codon was inserted before the variant splice site in order to produce a SH2B1 protein product (SH2B1 1-620) without the variable C-terminal region. This form of SH2B1 is isoform neutral. PC12 cells were transiently transfected with GFP, GFP-SH2B1β, or GFP-SH2B1 1-620 and treated with NGF to induce neuronal differentiation. In contrast to SH2B1α, SH2B1 1-620 enhances NGF-induced neuronal differentiation nearly to the same extent as SH2B1β (FIG. 3.9). This result suggests that there are properties of the unique SH2B1α C-terminal tail that are preventing nuclear entry of SH2B1 and the enhancement of NGF-induced neuronal differentiation.

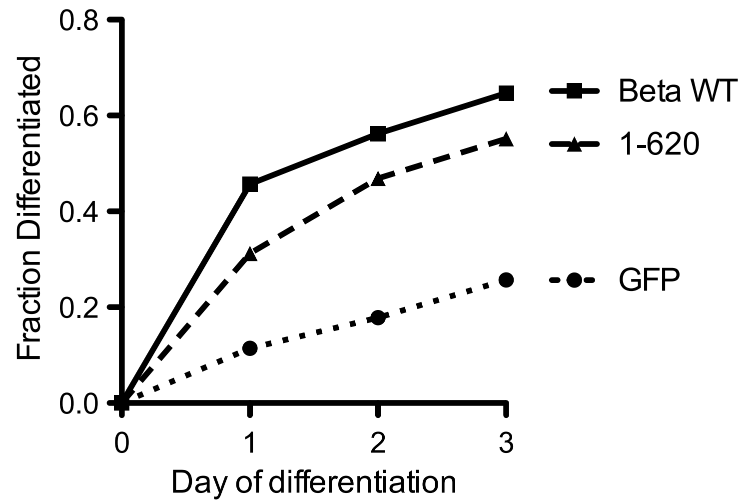


FIG. 3.9. SH2B1 1-620 enhances NGF-induced PC12 cell neurite outgrowth. PC12 cells expressing GFP, GFP-SH2B1β WT, or GFP-SH2B1 1-620 were treated with NGF (25 ng/ml) to induce differentiation. Neurites > 2X cell body length were scored as differentiated, representative assay.

Discussion

It has been shown previously that SH2B1β is able to enhance NGF-mediated differentiation and that the nuclear-cytoplasmic shuttling of SH2B1β appears to be required for this enhancement (62). In this report, we show that SH2B1α, in contrast to SH2B1β, does not enhance NGF-induced PC12 cell neuronal differentiation.

Although SH2B1α is capable of promoting the autophosphorylation of JAK2 and the insulin-stimulated tyrosyl phosphorylation of IRS2 to the same extent as SH2B1β, SH2B1α is unable to enhance NGF-induced neuronal differentiation. We showed that SH2B1α is unable to enter the nucleus with LMB treatment in 293T cells and does not enhance the expression of a sub-set of NGF-induced genes required for neuronal differentiation. We have also provided evidence that it is most likely the unique C-terminal tail of SH2B1α that prevents its ability to enhance neuronal differentiation.

These data suggest that SH2B1α does not play a role in neuronal differentiation (at least under our conditions in PC12 cells), which is supported by the fact that patients with *SH2B1* mutations that would only be present in the alpha isoform due to alternative splicing (A663V, V695M, and A723V) do not present with abnormal neurobehavioral phenotypes. While these patients are still obese and insulin resistant, which suggests that SH2B1α is important for

whole-body energy homeostasis and insulin sensitivity, they do not display the maladaptive behavior phenotype seen in patients with *SH2B1* human mutations that are present in the beta, gamma, or delta isoforms (all capable of enhancing NGF-induced neuronal differentiation). This suggests that there is another pathway or brain cell type that is affected by SH2B1α to influence whole-body energy homeostasis.

It will be important to understand how the unique C-terminal tail of SH2B1α acts to prevent SH2B1α from entering the nucleus, which may provide insight into how other proteins have their nuclear entry regulated. It seems likely that a great deal can be learned about the processes governing neuronal differentiation by investigating the signaling pathways and transcription factors that are differentially regulated by SH2B1α and SH2B1β. This insight may lead to the identification of targets for therapeutics that promote the positive regulation of whole-body energy homeostasis.

Chapter 4

Effects of human mutations on cellular actions of SH2B1 delta

Abstract

SH2B1 modulates signaling by a variety of ligands that bind to receptor tyrosine kinases or JAK-associated cytokine receptors, including receptors for leptin, insulin, growth hormone (GH), and nerve growth factor (NGF). Targeted deletion of *Sh2b1* in mice results in increased food intake, obesity, and insulin resistance, with an intermediate phenotype seen in heterozygous null mice on a high-fat diet. SH2B1 is expressed as four different isoforms: alpha, beta, gamma, and delta. Previously, *SH2B1* loss-of-function mutations have been identified in a large cohort of patients with severe early-onset obesity and the effects of these mutations on cellular actions of SH2B1β were investigated. Here, we identify additional mutations present in *SH2B1* from obese patients with disproportionate insulin resistance, including one mutation exclusive to the delta isoform. We show that SH2B1δ has a sub-cellular localization at the plasma membrane and nucleolus and that a bipartite NLS in the unique C-terminal tail of SH2B1δ is important for its nucleolar localization and enhancement of NGF-induced neurite outgrowth. We show that SH2B1δ with one of the human mutations does not enhance NGF-induced neurite outgrowth to same extent as SH2B1δ WT. However, we did not see a difference between the ability of SH2B1δ WT or

mutant to enhance JAK2 activation or enhance NGF-induced ERK 1/2 phosphorylation or expression of genes encoding MMP3 and MMP10.

Introduction

Recently, *SH2B1* has been identified as a human obesity gene. Humans with point mutations found in *SH2B1* exhibit severe early-onset obesity, extreme insulin resistance, and maladaptive behavior (112). The hyperphagia and maladaptive behavior seen in these patients hint at a possible role for SH2B1 in neuronal development. This is supported by the effects of neuronal SH2B1 in mice with a whole-body knockout of *Sh2b1*. *Sh2b1*$^{-/-}$ mice are obese and insulin resistant (18), yet when transgenic mice are created using a neuronal-specific enolase promoter to express SH2B1β mainly in neurons, the metabolic disorder phenotype is ameliorated (54).

The adaptor protein SH2B1 is expressed as four different isoforms (alpha, beta, gamma and delta) that arise due to alternative splicing (28, 29). The SH2B1 isoforms are identical in their sequence until variable splice sites produce unique C-terminal tails for each. While the beta isoform has been well characterized, there has not been much investigation into the 110-kDa delta isoform.

SH2B1δ has been seen to behave similarly to SH2B1β in promoting mitogenesis and cell survival in NIH 3T3 fibroblasts (28), and insulin receptor kinase activity in 3T3-L1 adipocytes (49). SH2B1β and γ have been reported to enhance nerve growth factor (NGF)-induced PC12 cell neuronal differentiation

(30, 31), in part by promoting the expression of a sub-set of NGF-induced genes required for differentiation (67). Nuclear-cytoplasmic shuttling of SH2B1β appears to be required for this enhancement of NGF-induced neuronal differentiation (62). Human mutations identified in *SH2B1* impair the ability of SH2B1β to enhance NGF-induced neuronal differentiation (112).

The unique C-terminal tail of SH2B1δ contains a putative bipartite nuclear localization signal (NLS) that may confer a unique sub-cellular localization for SH2B1δ compared to the primarily plasma membrane (PM) and cytosolic steady-state localization of SH2B1β. The human mutations reported previously to affect SH2B1β function are also present in SH2B1δ and, recently, more mutations have been identified that are only expressed in the alpha or delta isoforms. It appears that the expression of SH2B1δ is limited to brain tissues (112). Thus, it is important to better understand the cellular functions of SH2B1δ in neuronal processes and, particularly, how these are affected by the human mutations.

In this report, we show that the putative bipartite NLS is responsible for the nuclear/nucleolar localization of SH2B1δ and that intact NLS motifs are required for SH2B1δ to promote neuronal differentiation. We also characterize the effects of SH2B1δ WT or SH2B1δ with human mutations on NGF-induced neuronal differentiation, ERK phosphorylation, and gene expression.

Materials and Methods

Antibodies and Reagents. αFLAG (M2) (1:2000) (α = antibody) was from Sigma. αJAK2 p1007/1008 (1:1000) was from Upstate Biotechnology Inc. (Lake Placid, NY). αERK1/2 and αERK1/2 pT202/Y204 (1:1000) were from Cell Signaling Technology (Beverly, MA). IRDye800-conjugated anti-GFP, IRDye700 and IRDye800 conjugated anti-mouse and anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) were used at a dilution of 1:20,000. NGF and rat-tail collagen I were purchased from BD Bioscience (San Diego, CA).

Plasmids. Murine *SH2B1δ* (accession# AF380422) and rat *SH2B1β* (NM_001048180) were cloned into the pEGFP C1 vector (Clontech, Mountain View, CA) (described previously (62)) and pmCherry C1 vector (Clontech). Mutations were introduced using the QuickChange Mutagenesis kit (Stratagene). GFP-Nucleolin (114) was from Addgene (Cambridge, MA) (Addgene plasmid 28176). All constructs were verified by sequencing.

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 5% CO_2 in normal growth medium containing RPMI 1640 (ATCC), 10% heat-inactivated horse serum (Invitrogen), and 5% fetal bovine serum (Atlanta Biologicals). PC12 cells were transfected using a Gene

Pulser Xcell Electroporator (400V, 500μF) in a 0.4 cm cuvette (Bio-Rad). 293T cells (from Dr. O. A. MacDougald, University of Michigan, Ann Arbor, MI) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 8% calf serum (Atlanta Biologicals) and transfected using calcium phosphate.

Confocal Microscopy. Cell imaging was performed using an Olympus FluoView 500 laser scanning confocal microscope and FluoView version 5.0 software. Transfected 293T cells were plated on poly-d-lysine coated glass bottom dishes (MatTek, Ashland, MA) and imaged while in Ringer's buffer (10 mM HEPES, 155 mM NaCl, 2 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 10 mM NaH2PO4 and 10 mM glucose, pH 7.2). The plasma membrane was labeled with wheat germ agglutinin-conjugated Alexa Fluor 647 (Invitrogen).

Differentiation of PC12 cells. PC12 cells were grown on collagen-coated 10-cm^2 dishes then plated on collagen-coated 60-mm^2 dishes after transfection. 24 h post transfection, 25 or 50 ng/ml NGF was added to RPMI 1640 containing 5% horse serum, 1% FBS to induce PC12 cell differentiation. NGF-containing differentiation medium was refreshed two days later. The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200). The percent of GFP+ cells that were differentiated (neurite outgrowths >2 times the length of the cell body) was determined.

Cell lysis and immunoblotting. Cells were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample lysis buffer (50 mM Tris [pH 7.5], 1% SDS, 0.001% bromophenol blue, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA) and proteins were separated by SDS-PAGE.

Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with the indicated antibodies. Blots were then incubated with infrared dye-conjugated secondary antibodies and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

qPCR. Total RNA was isolated using Stat60 (Tel-Test) and cDNA was generated using TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). Gene expression was assessed using SYBR Green (Bio-Rad) and the Eppendorf Mastercycler ep (Hamburg, Germany). All readings were normalized to the expression of glyceraldehyde-3- phosphate dehydrogenase (GAPDH), which did not differ between the different transfected cells.

Results

C-Terminal bipartite NLS regulates nuclear/nucleolar localization of SH2B1δ. The N-terminal NLS (termed NLS1), which is common to all SH2B1 isoforms, has been shown to be required for SH2B1β to enter the nucleus (62). The unique C-terminal tail region of SH2B1δ contains two stretches of basic amino acids proximal to each other (termed NLS2 and NLS3, respectively) that closely resemble a bipartite nuclear localization sequence motif (115). A bipartite NLS motif has been shown to promote protein sub-cellular localization in the nucleolus (116). The nucleolus is a distinct region in the nucleus that is the main site of ribosome biosynthesis (117, 118) and RNA processing (119). The nucleolus has also been implicated in the regulation of the cell cycle (120), tumorigenesis (121) and viral replication (122). We first used confocal microscopy of living cells to look at the localization of cherry-SH2B1δ. In contrast to SH2B1β which localizes to the PM and cytosol, SH2B1δ WT was localized to the nucleolus; a small amount was also present in the PM, which was visualized with higher exposure duration. To investigate the role of the three NLS motifs in SH2B1δ sub-cellular localization, four basic amino acid residues in each NLS motif were mutated to Ala or Gly (FIG. 4.1).

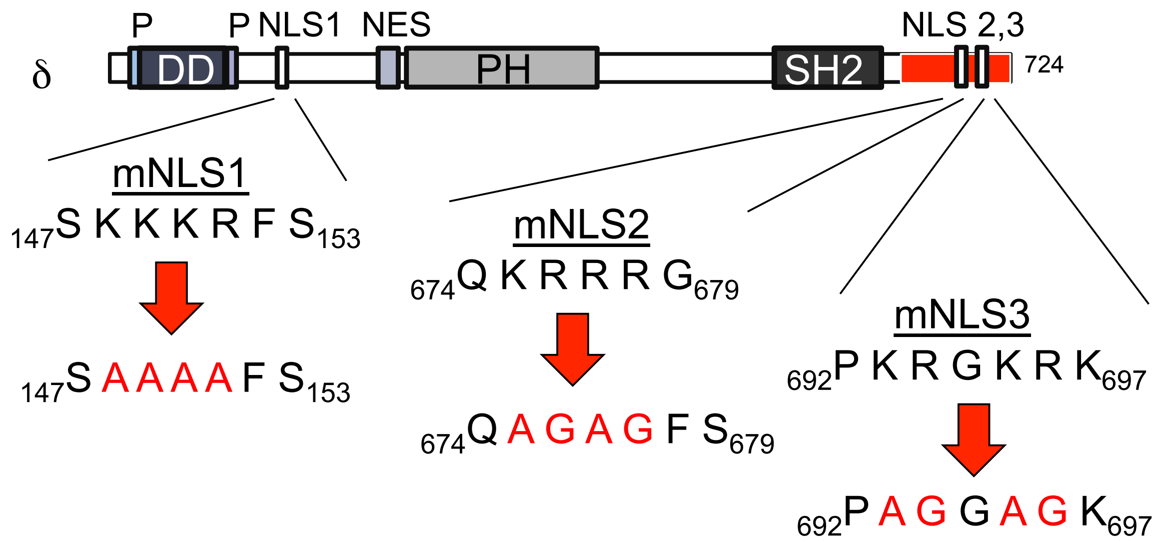


FIG. 4.1. Schematic of SH2B1δ NLS mutations. P, proline-rich region; DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain. Lys or Arg from the WT NLS sequences (black) were mutated to Ala or Gly (red).

An intact NLS1 has also been shown to be required for SH2B1β to go to both the nucleus the PM (65). Thus, we would predict that any SH2B1δ construct with a mutated NLS1 would not localize at the PM. Since SH2B1δ is the only SH2B1 isoform with a C-terminal bipartite NLS motif and is the only isoform seen to localize at the nucleolus, we also predicted that disruption of NLS2 and NLS3 would prohibit nucleolar localization of SH2B1δ. The steady-state sub-cellular localization of each SH2B1δ construct was determined in living cells using confocal microscopy. Both SH2B1δ mNLS1 and mNLS2 were localized at the nucleolus, similar to WT, but SH2B1δ mNLS1 was no longer present at the PM. SH2B1δ mNLS3 is seen localized at the nucleoplasm (as opposed to specifically at the nucleolus) and PM to a greater extent than WT. SH2B1δ mNLS2/3 was excluded from the nucleus but present at the PM. SH2B1δ mNLS1/2/3 appeared to potentially be toxic when overexpressed as the cells looked unhealthy when imaged and displayed a variable sub-cellular localization (seen with diffuse or aggregate localization) (FIG. 4.2).

These results suggest that an intact NLS1 is required for SH2B1δ to localize to the PM and that when NLS1 is intact, only one of the C-terminal NLS motifs is required for steady-state nuclear/nucleolar localization of SH2B1δ. However, these results also indicate that an intact bipartite NLS is not required for nucleolar steady-state localization of SH2B1δ as SH2B1δ mNLS2 and mNLS3 exhibited steady-state localization in the nucleolus. This may suggest that only one part of the C-terminal partite NLS motif is required for nucleolar

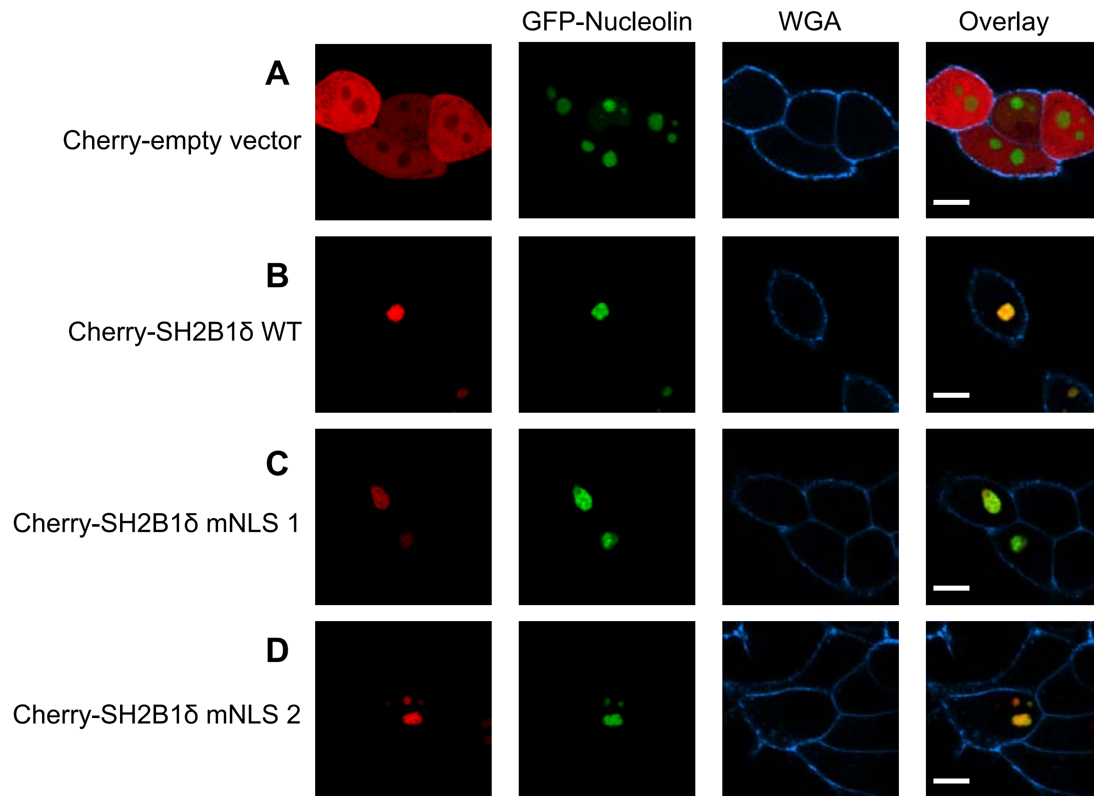


FIG. 4.2. SH2B1δ requires NLS1 to localize at the PM and both components of the bipartite NLS to localize at the nucleolus. 293T cells transiently expressing Cherry-SH2B1 and GFP-Nucleolin (protein marker of the nucleolus) were stained with the plasma membrane wheat germ agglutinin Alexa Fluor 647 and imaged using fluorescent confocal microscopy. Cherry = red, GFP-Nucleolin = green, Wheat Germ Agglutinin (WGA) = blue. Scale bars: 10 µm. (**A**) Cherry-empty vector depicts a diffuse sub-cellular localization, but not at the nucleolus. (**B**) Cherry-SH2B1δ WT appears localized at the nucleolus. (**C**) Cherry-SH2B1δ mNLS 1 localizes at the nucleolus. (**D**) Cherry-SH2B1δ mNLS2 appears localized at the nucleolus.

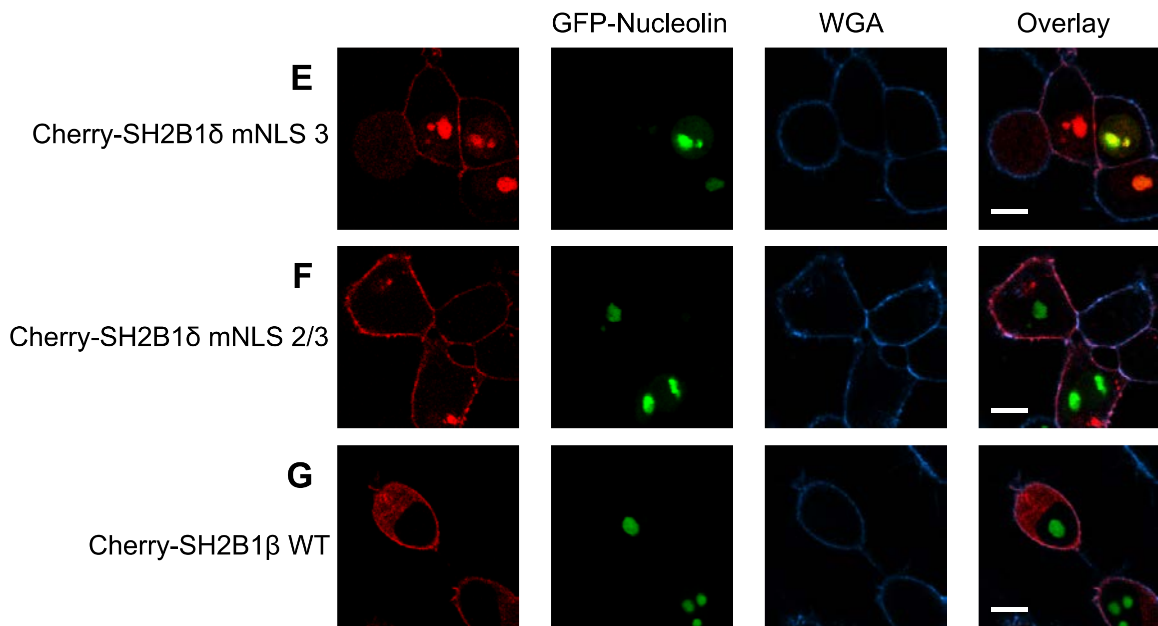


FIG. 4.2. SH2B1δ requires NLS1 to localize at the PM and both components of the bipartite NLS to localize at the nucleolus. 293T cells transiently expressing Cherry-SH2B1 and GFP-Nucleolin (protein marker of the nucleolus) were stained with the plasma membrane (pm) dye wheat germ agglutinin Alexa Fluor 647 and imaged using fluorescent confocal microscopy. Cherry = red, GFP-Nucleolin = green, Wheat Germ Agglutinin (WGA) = blue. Scale bars: 10 µm. (**E**) Cherry-SH2B1δ mNLS3 depicts a nucleolar and pm sub-cellular localization. (**F**) Cherry-SH2B1δ mNLS 2/3 localizes at the pm. (**G**) Cherry-SH2B1β WT localizes at the pm and cytosol.

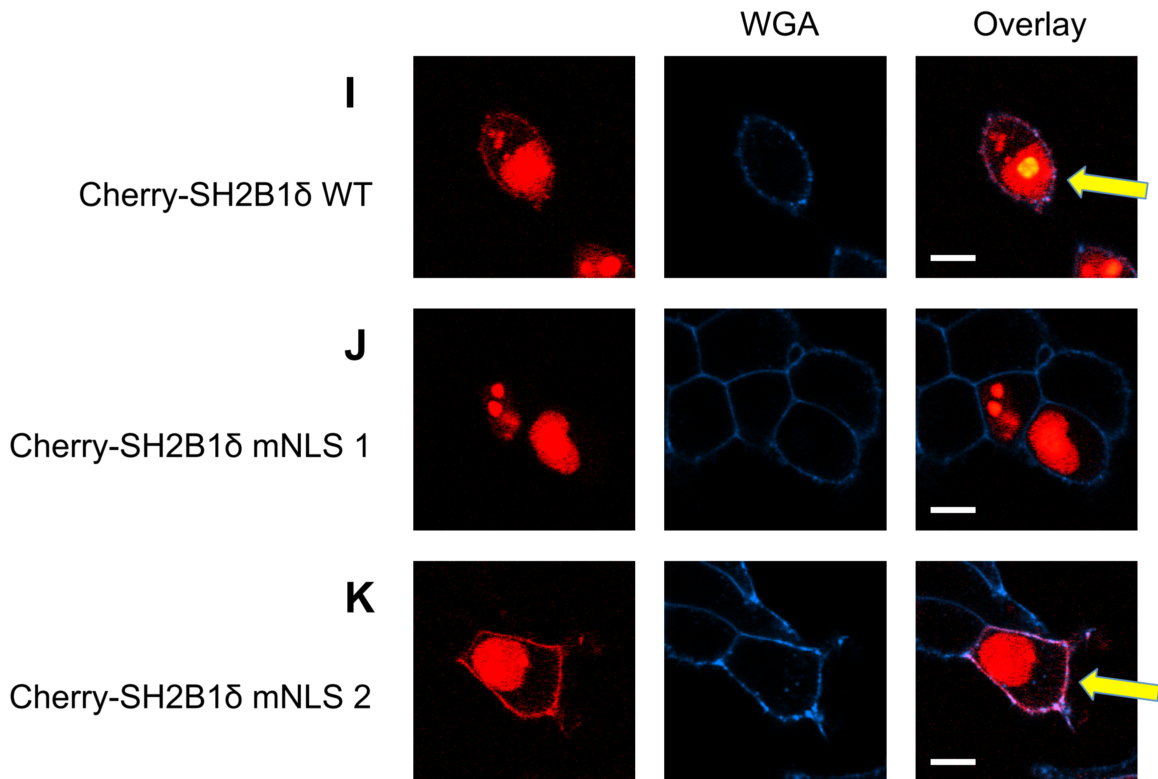


FIG. 4.2. SH2B1δ requires NLS1 to localize at the PM and both components of the bipartite NLS to localize at the nucleolus. 293T cells transiently expressing Cherry-SH2B1 and GFP-Nucleolin (protein marker of the nucleolus) were stained with the plasma membrane wheat germ agglutinin Alexa Fluor 647 and imaged using fluorescent confocal microscopy. Cherry = red, GFP-Nucleolin = green, Wheat Germ Agglutinin (WGA) = blue. Scale bars: 10 µm. (**I**) Cherry-SH2B1δ WT appears localized at the pm when imaged at a high exposure. (**J**) Cherry-SH2B1δ mNLS 1 was not detected at the pm even when imaged using a high exposure. (**K**) Cherry-SH2B1δ mNLS 2 appears localized at the pm when imaged at a high exposure. Yellow arrows indicate pm localization determined by a purple signal representing co-localization of Cherry-SH2B1δ and WGA.

localization but because SH2B1δ mNLS 2/3 was excluded from the nucleus entirely, there may be still unidentified regions of the SH2B1δ C-terminal tail responsible for the direct nucleolar localization of SH2B1δ.

Intact NLS motifs are required for SH2B1δ to enhance NGF-induced neuronal differentiation. To assess whether SH2B1δ, like SH2B1β, enhances NGF-induced neuronal differentiation, PC12 cells were transiently transfected with either empty-vector Cherry plasmid, or Cherry-tagged SH2B1β WT or SH2B1δ WT and treated with NGF to initiate differentiation. The PC12 cell line has been commonly used as a model system to investigate the mechanisms of NGF-mediated neuronal differentiation and NGF signal transduction (86). RNAi experiments have shown that SH2B1 is required for NGF-induced neurite outgrowth (67). PC12 cells are derived from a solid rat pheochromocytoma tumor and, when treated with NGF, differentiate into a neuronal type cell with properties similar to those of sympathetic neurons (87). When PC12 cells are treated with NGF, they stop proliferating and initiate a differentiation process in which they exhibit cellular hypertrophy, produce neurite outgrowths, express neuronal-specific markers, and become electrically excitable (86, 87). Differentiated sympathetic-like PC12 neuronal cells are able to form synapses with primary neurons (88), supporting the biological relevance of this *in vitro* cell line. SH2B1δ WT, like SH2B1β, promoted a large enhancement of NGF-induced neurite outgrowth (FIG. 4.3) (a cell was scored as differentiated

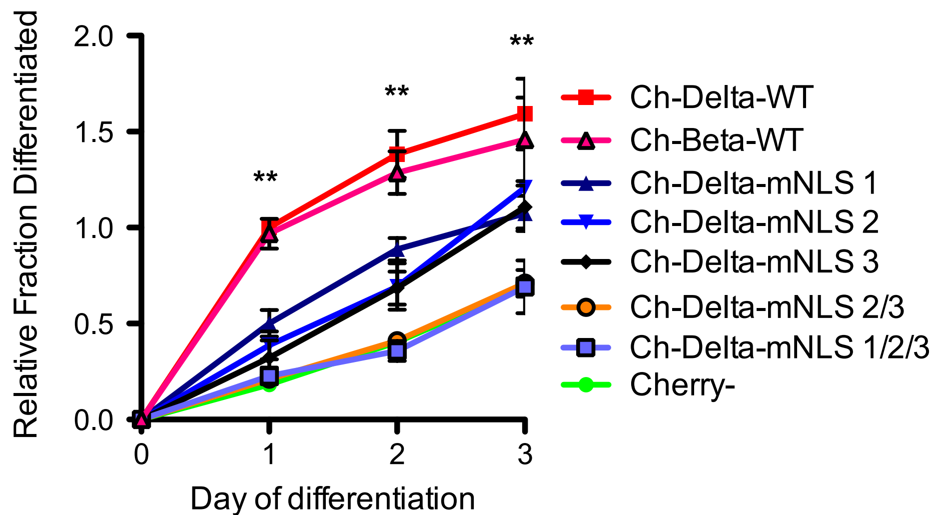


FIG. 4.3. SH2B1δ WT, but not with a mutated NLS, enhances NGF-induced PC12 cell differentiation to the same extent as WT. PC12 cells transiently expressing cherry-tagged SH2B1δ or SH2B1βWT, or SH2B1δ a mutant NLS were treated with NGF (25 ng/ml) to induce differentiation. Percent differentiated values were normalized against WT at day 1 of differentiation (means ± SEM). The fractions of GFP+ cells that were differentiated at each day of differentiation were counted (a cell was scored as differentiated if the length of its neurite outgrowths were greater than two-times the length of the cell body). Percent differentiated values were normalized against SH2B1δ WT at day 1 of differentiation (means ± SEM). All mutants impaired the rate of NGF-induced neuronal differentiation compared to SH2B1δ and SH2B1β WT. SH2B1δ and SH2B1β WT vs. others, **$p < 0.0001$, 1-way ANOVA with Dunnett's post-test, n = 3-4 separate experiments. NOTE: SH2B1δ and SH2B1β WT are statistically significantly different than SH2B1δ mNLS 1, mNLS 2, or mNLS 3 at day 3 of differentiation.

if the length of its neurite outgrowths were greater than two-times the length of the cell body).

Since nuclear shuttling is required for SH2B1β to enhance NGF-induced neuronal differentiation, we predicted that SH2B1δ with NLS mutations that exclude it from the nucleus would also fail to enhance NGF-induced neuronal differentiation. To determine whether sub-cellular localization of SH2B1δ is important for NGF-induced neuronal differentiation, PC12 cells were also transfected with Cherry-tagged SH2B1δ mNLS1, SH2B1δ mNLS2, SH2B1δ mNLS3, SH2B1δ mNLS2/3, or SH2B1δ mNLS1/2/3 and treated with NGF to initiate differentiation. SH2B1δ mNLS1, mNLS2, and mNLS3 were able to enhance differentiation but not to the same extent as WT. SH2B1δ WT mNLS2/3 and mNLS1/2/3 failed to promote NGF-induced neuronal differentiation (FIG 4.3). These results suggest that mutations that exclude SH2B1δ from the nucleus inhibit NGF-induced differentiation while mutations that do not exclude SH2B1δ from the nucleus are still able to promote NGF-induced neuronal differentiation, although at an impaired rate compared to WT.

SH2B1δ with human mutations do not enhance NGF-induced neuronal differentiation to the same extent as SH2B1δ WT. The human mutations identified previously to affect the function of SH2B1β are also present in SH2B1δ. Since the publication of the initial report to characterize human mutations in SH2B1, more mutations in SH2B1 from patients with early onset obesity and extreme insulin resistance have been identified. Most of the identified mutations are common to all SH2B1 isoforms. These include the

previously reported P90H, T175N, P322S, F344LfsX20, and the novel A484T (which is possibly a single-nucleotide polymorphism) and T546A mutations. However, some mutations have been identified that are present in only SH2B1 alpha or delta transcripts. In SH2B1δ, these mutations are R680C (an A663V mutation in the alpha isoform) and P712P. The P712P mutation (which is V695M in the alpha isoform) contains a nucleotide change that does not lead to an actual amino acid change in SH2B1δ. The resulting codon still produces a Pro (FIG. 4.4).

To assess the effects of the human mutations in SH2B1δ on NGF-induced neurite outgrowth, PC12 cells were transiently transfected with GFP, GFP-SH2B1δ WT, or GFP-SH2B1δ with a human mutation. SH2B1δ with the human mutations P90H, T175N, P322S, T546A, and R680C (which is located in the C-terminal tail and is unique to SH2B1δ) all show impaired enhancement of NGF-induced neuronal differentiation, while SH2B1δ A484T and P712P enhanced NGF-induced neuronal differentiation to a similar extent as SH2B1δ WT (FIG. 4.5), indicating that an alteration alone in the nucleotide sequence of SH2B1 without a change in the protein sequence is not sufficient to cause dysfunction of SH2B1.

SH2B1δ enhances ERK phosphorylation in response to NGF. A sustained ERK phosphorylation in response to NGF is associated with PC12 cell differentiation while more transient ERK phosphorylation (as seen in response to EGF) is associated with PC12 cell proliferation (93-96). We therefore looked

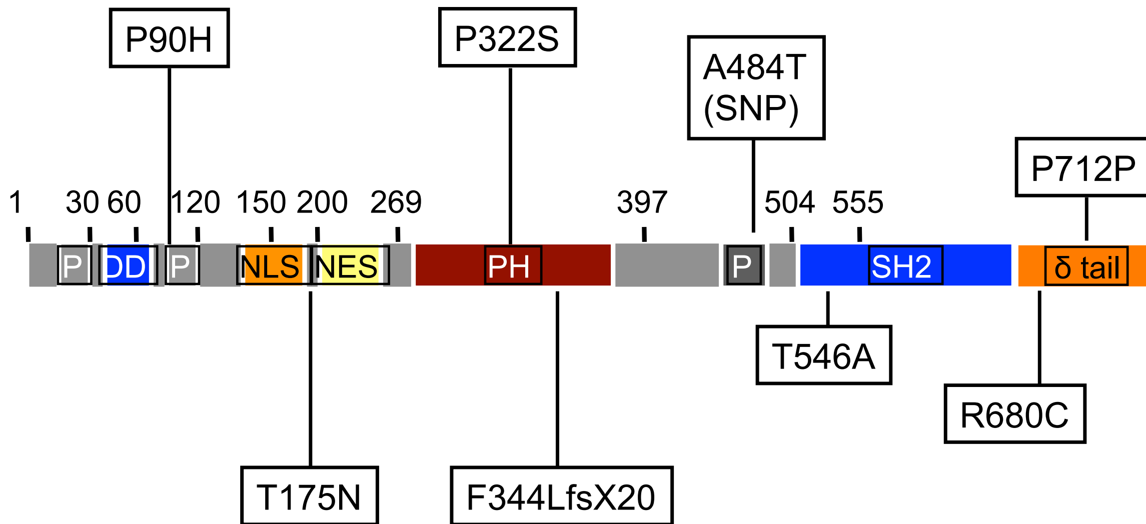


FIG. 4.4. Identification of human mutations in SH2B1δ. P, proline-rich region; DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain, unique SH2B1δ C-terminal tail in orange. SH2B1δ mutations are shown in boxes.

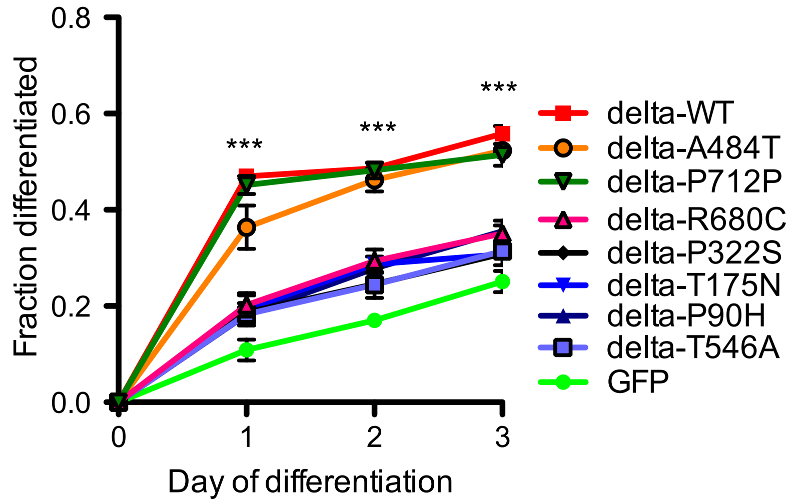


FIG. 4.5. SH2B1δ with disease-causing human mutations do not enhance NGF-induced PC12 cell differentiation to the same extent as WT. PC12 cells transiently expressing GFP-tagged WT or mutant SH2B1δ were treated with NGF (25 ng/ml) to induce differentiation. The fractions of GFP+ cells that were differentiated at each day of differentiation were counted with means ± SEM shown (a cell was scored as differentiated if the length of its neurite outgrowths were greater than two-times the length of the cell body). All mutants impaired the rate of NGF-induced neuronal differentiation compared with WT. SH2B1δ WT, A484T, and P712P vs. others, ***$p < 0.0001$, 1-way ANOVA with Bonferroni's multiple comparison post-test, $n = 3$ separate experiments.

to see whether SH2B1δ altered ERK phosphorylation and whether the human mutations impaired ERK phosphorylation in response to NGF. To carry out these studies, PC12 cells stably expressing GFP, GFP-SH2B1β WT, GFP-SH2B1δ WT, GFP-SH2B1δ P322S, or GFP-SH2B1δ R680C were created (FIG. 4.6).

The stably transfected PC12 cells were treated with NGF (25 ng/ml) for 5 and 30 minutes. Cell lysates were immunoblotted with a phospho-specific antibody that recognizes the doubly phosphorylated, active forms of ERKs 1/2. SH2B1β did not affect NFG activation of ERKs as reported previously (31). In contrast to SH2B1β, SH2B1δ-expressing cells exhibited an increased and prolonged level of NGF-dependent ERK phosphorylation compared to GFP-expressing cells (FIG. 4.7). To see if the human mutations impair the ability of SH2B1δ to enhance NGF-induced ERK phosphorylation, GFP-SH2B1δ WT, P322S and R680C expressing PC12 cells were treated with NGF (25 ng/ml) for 5, 120, and 240 minutes. At each time point, SH2B1δ P322S and R680C enhanced ERK phosphorylation to the same extent as SH2B1δ WT (FIG. 4.8).

SH2B1δ enhances NGF-induced gene expression. Overexpression of SH2B1β has been shown to enhance the expression of a sub-set of NGF-induced genes in PC12 cells. This enhancement of gene expression is associated with enhancement of neuronal differentiation (67). Among the sub-set of NGF-induced genes, *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, were confirmed to be dependent on SH2B1 for their enhanced

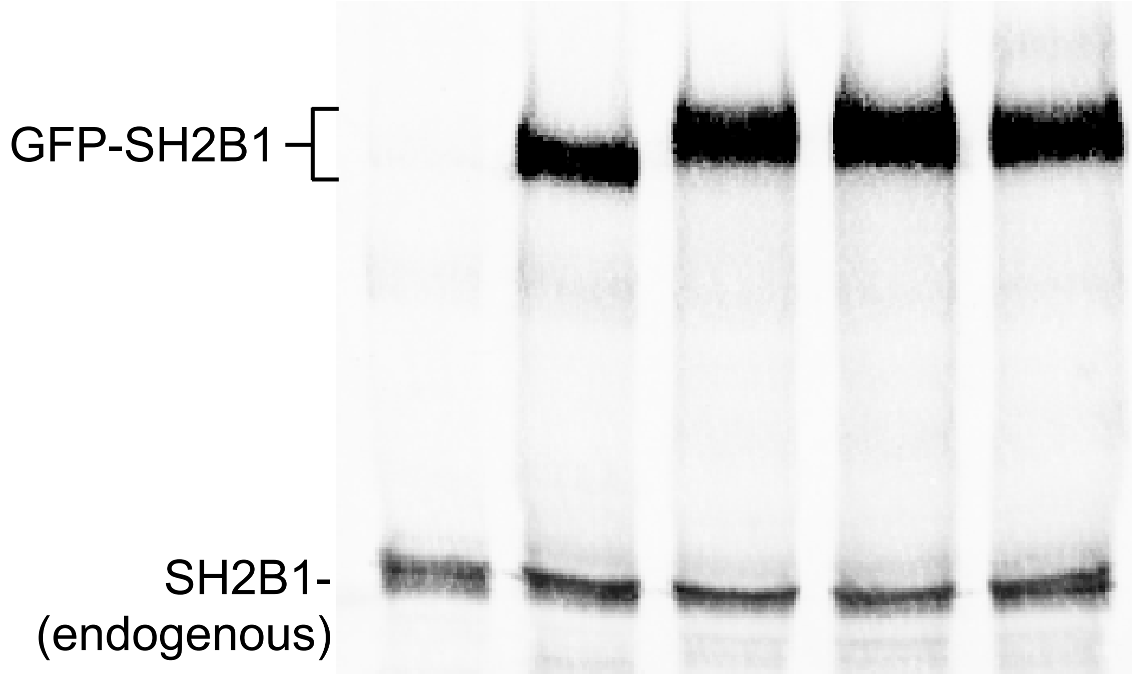


FIG. 4.6. PC12 cells stably express GFP-SH2B1β, or GFP-SH2B1δ WT, P322S, or R680C. Proteins in lysates from PC12 cells stably expressing GFP, GFP-SH2B1β, GFP-SH2B1δ WT, GFP-SH2B1δ P322S, or GFP-SH2B1δ R680C were separated by SDS-PAGE and immunoblotted with α-SH2B1. Overexpressed GFP-SH2B1 and endogenous SH2B1 are noted.

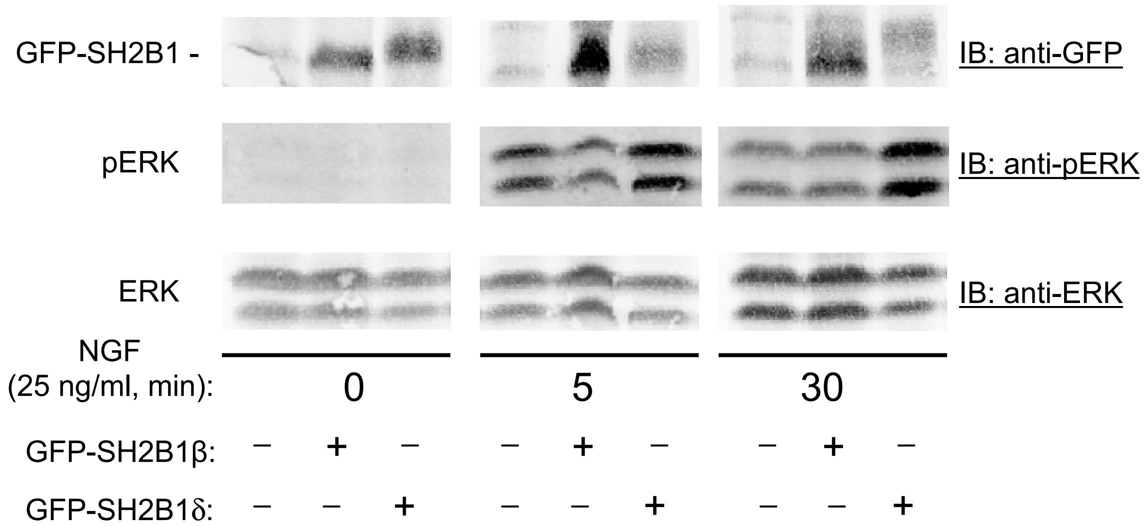


FIG. 4.7. SH2B1δ, but not SH2B1β, enhances NGF-induced ERK phosphorylation. Proteins in lysates from PC12 cells stably expressing GFP, GFP-SH2B1β, or GFP-SH2B1δ and treated with NGF (25 ng/ml) for the indicated time were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-pERK 1/2 (middle panel), or α-ERK 1/2 (bottom panel).

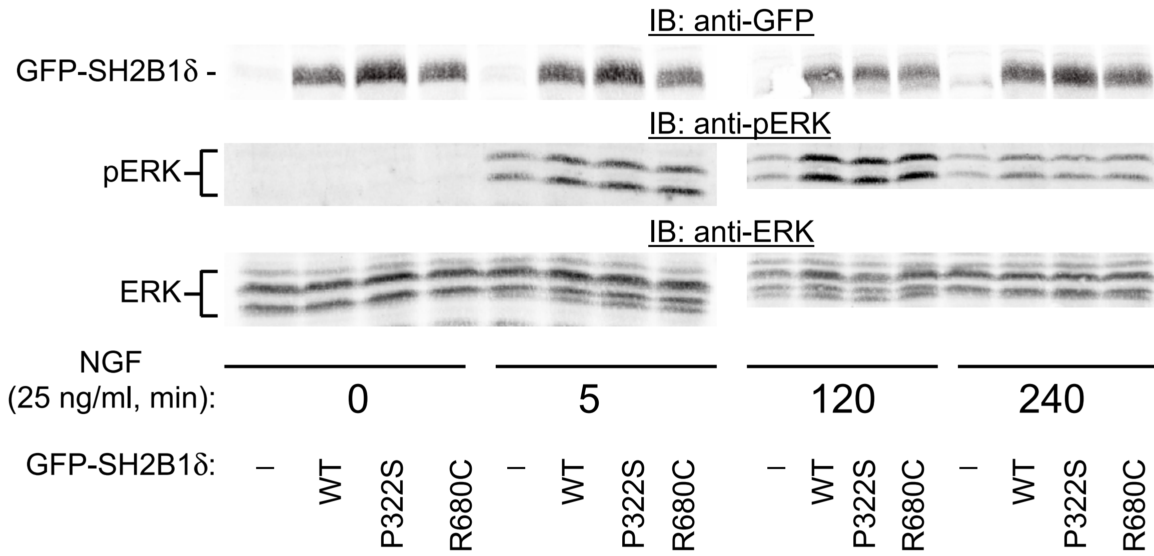


FIG. 4.8. SH2B1δ P322S and R680C enhance NGF-induced ERK phosphorylation to the same extent as SH2B1δ WT. Proteins in lysates from PC12 cells stably expressing GFP, GFP-SH2B1δ WT, P322S, or R680C and treated with NGF (25 ng/ml) for the indicated time were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-pERK 1/2 (middle panel), or α-ERK 1/2 (bottom panel).

expression. This cluster of genes is important for extra-cellular matrix degradation and neurite extension associated with neuronal differentiation (68-71). Because SH2B1δ, like SH2B1β, enhances neurite outgrowth, we investigated whether overexpression of SH2B1δ, like overexpression of SH2B1β, enhances NGF-induced expression of *Plaur*, *Mmp3*, and *Mmp10*. The PC12 cell lines expressing GFP, GFP-SH2B1β WT, or GFP-SH2B1δ WT were treated for six hours with NGF (25 ng/ml) and relative levels of MMP3, MMP10, and uPAR mRNA were assessed by qPCR. Consistent with previous results, SH2B1β enhanced NGF-induced expression for all three genes. SH2B1δ also enhanced expression of these three genes, however levels of enhancement of NGF-induced gene expression from all conditions were highly variable and there was no significant difference detected in the ability to enhance NGF-induced gene expression between WT and SH2B1δ P322S and R680C (FIG. 4.9). This suggests that SH2B1δ may be acting similarly to SH2B1β to regulate gene expression in the nucleus in response to NGF but more experiments are required to determine whether SH2B1δ P322S and R680C actually impair SH2B1δ enhancement of NGF-induced gene expression.

SH2B1δ enhances JAK2 autophosphorylation. SH2B1β was originally identified as a binding partner for the receptor-associated tyrosine kinase JAK2 (22). Many cytokine and growth factor receptors utilize JAK2 to mediate their signal transduction, including the receptors for growth hormone (GH) and leptin. When co-expressed with JAK2, SH2B1β has been shown to

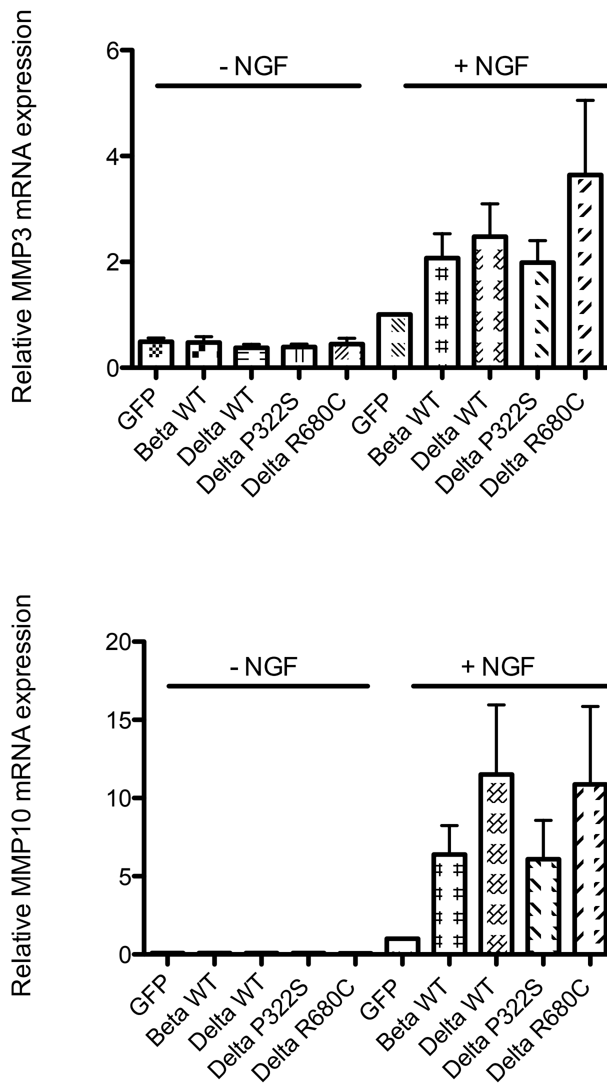


FIG. 4.9. Overexpression of SH2B1δ WT or with a human mutation promotes NGF-induced expression of MMP3, and MMP10 mRNA expression. PC12 cells stably expressing GFP, or GFP-tagged SH2B1β WT, SH2B1δ WT, SH2B1δ P322S, or SH2B1δ R680C were NGF (25 ng/mL) for 6 hours. Relative mRNA levels were determined using qPCR, normalized to levels of GAPDH mRNA and then to the mRNA levels in NGF-treated GFP control cells. Means ± SEM (n=3). Due to high variability between assays none the values are statistically significantly different from each other. More experiments are required.

enhance JAK2 tyrosine kinase signaling activity. This requires an intact SH2 domain of SH2B1β to bind to phospho-tyrosine 813 of JAK2 (40, 113). To determine whether SH2B1δ, like SH2B1β, activates JAK2, 293T cells were transiently transfected with cDNA encoding Flag-tagged JAK2 and GFP-tagged SH2B1β WT or SH2B1δ WT. JAK2 activity was assessed by blotting with α-pJAK2 Y1007/1008, which recognizes the active form of JAK2. SH2B1δ WT is able to enhance JAK2 activity, similarly to what is seen when JAK2 is co-expressed with SH2B1β (FIG. 4.10).

Next we tested whether human mutations identified in *SH2B1* impaired the ability of SH2B1δ to activate JAK2. SH2B1δ with human mutations P90H, T175N, P322S, A484T, T546A, R680C, and P712P enhanced JAK2 activity to a similar extent as SH2B1δ WT (FIG. 4.11). All mutants expressed at the expected size and at similar levels. While subtle differences in the ability to activate JAK2 may be masked in this assay by overexpression of SH2B1δ and JAK2, these results are consistent with both SH2B1β and SH2B1α with human mutations enhancing JAK2 activity to the same extent as WT (unpublished observations, Chapter 2 and 3 of this dissertation).

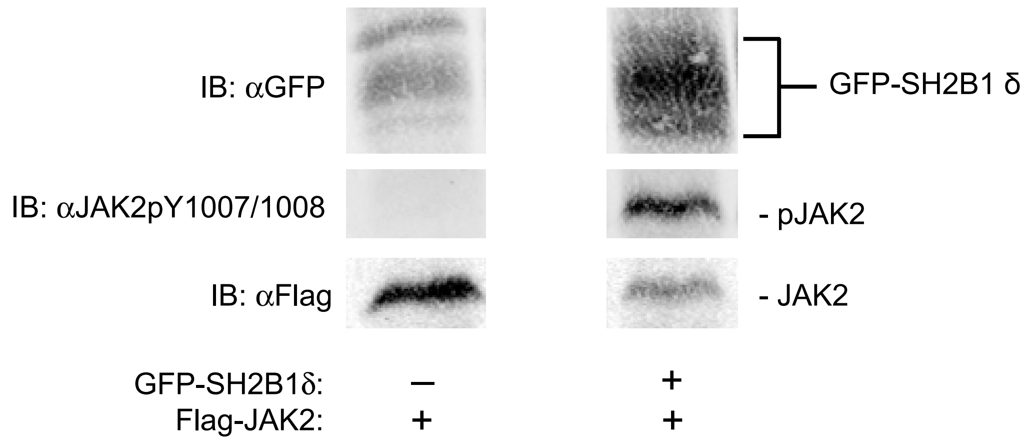


FIG. 4.10. SH2B1δ enhances JAK2 phosphorylation. Proteins in lysates from 293T cells transiently expressing Flag-JAK2 and GFP-SH2B1δ were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-JAK2pY1007 (middle panel), or α-JAK2 (bottom panel).

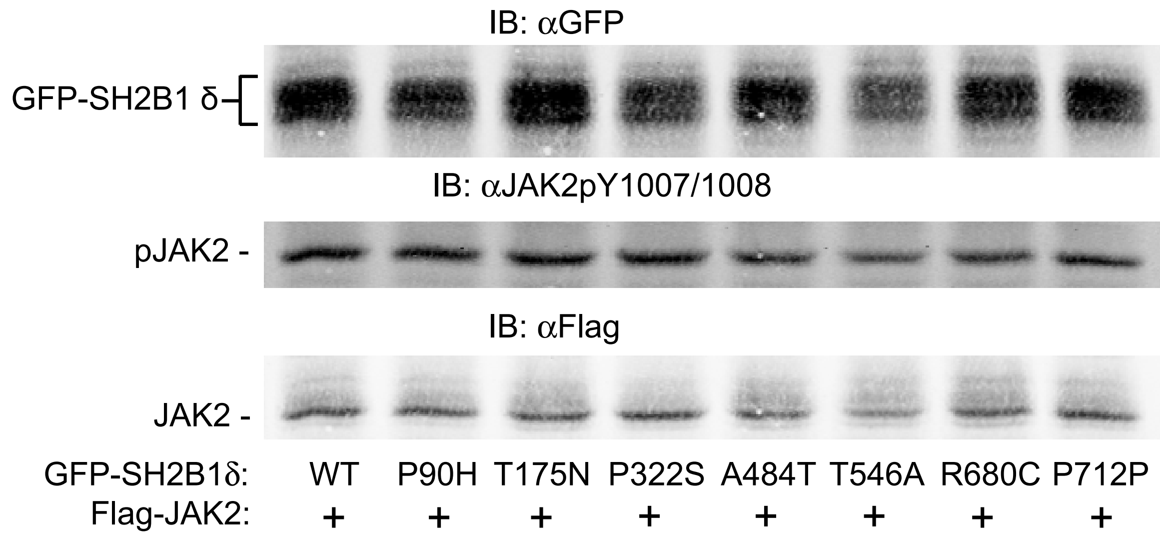


FIG. 4.11. SH2B1δ with a human mutation enhances JAK2 phosphorylation to the same extent as SH2B1δ WT. Proteins in lysates from 293T cells transiently expressing Flag-JAK2 and GFP-SH2B1δ WT, P90H, T175N, P322S, A484T, T546A, R680C, and P712P were separated by SDS-PAGE and immunoblotted with α-GFP (top panel), α-JAK2pY1007 (middle panel), or α-JAK2 (bottom panel).

Discussion

We have shown previously that SH2B1β enhances NGF-mediated neurite outgrowth of PC12 cells and that the nuclear-cytoplasmic shuttling of SH2B1β appears to be required for this enhancement. In contrast to SH2B1β, which is widely expressed, previous results indicate SH2B1δ may have its expression limited to brain tissues (112). Here, we show that SH2B1δ has a steady-state sub-cellular localization at the nucleolus and PM. Activation of ribosome biosynthesis at the nucleolus is sufficient to cause cellular hypertrophy and neurite outgrowth in neuronal cells (123). Together, these findings suggest that nucleolar SH2B1δ may be playing a role in neuronal differentiation.

We confirmed that the putative bipartite NLS in the unique C-terminal tail of SH2B1δ is responsible for the steady-state nuclear/nucleolar sub-cellular localization of SH2B1δ. We also showed that similar to SH2B1β (65), the NLS1 of SH2B1δ is required for PM localization. We then showed that mutating NLS 1, 2, or 3 partially prevented SH2B1δ enhancement of NGF-induced neurite outgrowth, suggesting that PM localization and nuclear shuttling of SH2B1δ are required to fully enhance NGF-induced PC12 cell differentiation. These data are consistent with the notion that the actions of SH2B1 in the nucleus and at the PM are critical for the promotion of NGF-induced neuronal differentiation. While it is clear that the bipartite NLS of SH2B1δ directs its steady-state localization at the

nucleus, it remains unclear which region of the SH2B1δ C-terminal tail is directly responsible for localizing SH2B1δ at the nucleolus. SH2B1δ mNLS2 and mNLS3 still localized at the nucleolus, but mNLS2/3 was excluded from nucleus. No SH2B1δ cDNA construct tested displayed a nuclear but non-nucleolar SH2B1δ localization. RNA-binding motifs (stretches of aromatic amino acids) and glycine/arginine-rich (GR) domains have been implicated in targeting nuclear proteins to the nucleolus (124). The SH2B1δ C-terminal tail contains a Trp-rich region as well as GR segments within the bipartite NLS (28). Analysis of sub-nuclear localization of a series of deletion mutations should help to elucidate the region of the SH2B1δ C-terminal tail responsible for localizing SH2B1δ at the nucleolus.

We also describe the ability of SH2B1δ to enhance NGF-induced PC12 cell neuronal differentiation and the inability of mutant SH2B1δ to enhance the rate of differentiation to the same extent as WT. SH2B1β and SH2B1δ promoted similar rates of enhanced NGF-induced neuronal differentiation. As more and more subjects have their *SH2B1* gene sequenced, it appears that A484T may just be a single nucleotide polymorphism (SNP) and not a disease-causing mutation (I. Sadaf Farooqi, personal communication). Our results are consistent with A484T being a non-disease causing SNP as SH2B1δ A484T was able to enhance differentiation to a similar extent as WT. The mutations shared among all isoforms of SH2B1 (P90H, T175N, P322S, T546A) and the delta-specific mutation, R680C, exhibited impaired enhancement of NGF-induced neuronal

differentiation, similar to what has been reported for human mutations in the beta isoform (112).

Nuclear accumulation is impaired in SH2B1β with human mutations, possibly explaining why those mutants are unable to promote neuronal differentiation to the same extent as SH2B1β WT. In contrast, SH2B1δ with a human mutation is constitutively present in the nucleus/nucleolus and at the PM (data not shown). Thus, a similar explanation is unlikely to hold for why SH2B1δ mutations impair its ability to enhance NGF-induced neurite outgrowth. Thus, we assayed for SH2B1δ activation of ERKs 1/2, which is critical to PC12 cell differentiation. In contrast to SH2B1β, SH2B1δ promoted an enhanced and sustained level of ERK 1/2 phosphorylation. Thus, the enhanced response could be due to an enhanced ERK 1/2 response. However, SH2B1δ P322S and R680C promoted a similar level of ERK 1/2 phosphorylation as SH2B1δ WT, so we cannot explain the impaired NGF-induced neuronal differentiation seen with SH2B1δ mutants by an impaired ERK 1/2 activation.

SH2B1β has been shown to enhance the expression of a sub-set of NGF-induced genes that are important for neurite outgrowth. We show that SH2B1δ is able to promote an enhancement of expression of the NGF-induced genes *Mmp3*, *Mmp10* and *Plaur*. Surprisingly, SH2B1δ P322S and R680C promoted a similar level of NGF-induced gene expression as WT. More experiments are needed to provide statistically significant results. These results suggest that human mutations in SH2B1δ might impair NGF-induced expression of other genes or impair NGF-induced differentiation by a different mechanism.

Unlike SH2B1β with human mutations that exhibits impaired nuclear accumulation, a mechanism by which SH2B1δ with a human mutation leads to impaired enhancement of neuronal differentiation has yet to be identified. It may be that the human mutations are affecting the ability of SH2B1δ to regulate a signaling pathway or cell process not yet identified to be regulated by SH2B1, most likely a signaling event downstream or independent of ERKs. To fully understand the role SH2B1 plays in the regulation of neuronal differentiation, it is crucial to identify binding partners of SH2B1 in the nucleus of neurotrophin-stimulated cells.

In summary, SH2B1δ has been identified as a nucleolar protein that is a positive regulator of NGF-induced neuronal differentiation, ERK phosphorylation, and gene expression. Nuclear localization of SH2B1δ is important for the enhancement of neuronal differentiation. Human mutations identified in *SH2B1* and introduced into SH2B1δ exhibit an impaired ability to promote NGF-induced differentiation compared to WT, yet are able to enhance ERK phosphorylation and gene expression in response to NGF to a similar extent as SH2B1δ WT. Thus, the mechanism by which the human mutations impair the ability of SH2B1δ to enhance neurite outgrowth is unknown.

Chapter 5

The adapter protein SH2B1 and growth hormone-induced gene expression

Abstract

Metabolic effects of growth hormone (GH) in adipose tissue have been shown to be mediated, at least in part, by changes in gene expression. The adapter protein SH2B1 is a signaling molecule for many cytokines and growth factors, including GH. SH2B1 is recruited to the GH- activated form of the GH receptor-associated tyrosine kinase JAK2, resulting in its phosphorylation by JAK2 on tyrosines and on serine/threonines by other kinases. SH2B1 has been implicated in GH regulation of the actin cytoskeleton. Recently, our laboratory has identified both a nuclear localization signal (NLS) and nuclear export sequence (NES) in SH2B1. SH2B1 constitutively shuttles in and out of the nucleus, even though its steady-state sub-cellular localization is mostly in the plasma membrane and cytosol. In PC12 neuronal cells stimulated with nerve growth factor (NGF), over-expression of wild-type SH2B1, but not SH2B1 that is incapable of entering the nucleus (SH2B1 mNLS), enhances the expression of a sub-set of NGF-induced genes. Together, these data led us to hypothesize that SH2B1 regulates GH-induced gene expression in adipocytes. To test this, cultured 3T3-F442A adipocytes with endogenous SH2B1 knocked-down by RNAi and control cells were stimulated with GH for 1 h and a sub-set of genes that depend on SH2B1 for their full GH-

induced expression was identified by microarray analysis. Of the 16,361 total genes on the array, 611 were up-regulated by GH at least 60%; of those genes, 247 appeared to depend on SH2B1 for their full GH-induced expression. GH-dependent expression of *Apelin*, *Cish* and *Arid5a* genes and their decreased GH-induced expression in cells with decreased levels of SH2B1 were confirmed by qPCR. The re-introduction of SH2B1 in 3T3-F442A preadipocytes in which endogenous SH2B1 is knocked-down is able to rescue GH-induced expression of *Cish* and *Arid5a*. Overexpression of SH2B1, but not SH2B1 mNLS, enhances GH-induced expression of these genes. These results are consistent with SH2B1 being required for maximal expression of a sub-set of GH-dependent genes. Further, nuclear localization of SH2B1 may be required for SH2B1 enhancement of expression of a subset of GH-dependent genes.

Introduction

Growth hormone (GH) regulates multiple physiological functions, including body growth and metabolism. Among its best characterized metabolic actions are its ability to decrease fat and increase lean body mass. GH regulation of energy homeostasis and metabolism involves multiple tissues including skeletal muscle, liver, and adipose tissue. GH is classically known as a physiological antagonist of insulin action (reviewed in (125)). The opposing nature of GH and insulin is evident by their contrasting influences on adipose cell metabolism (126). When energy produced from nutritional intake surpasses the body's rate of energy expenditure, adipose tissue serves as a repository for excess energetic metabolites. Insulin stimulation causes adipocytes to take up glucose and free fatty acids (FFA) with conversion to triglycerides for storage, a process known as lipogenesis. GH, on the other hand, has been seen *in vivo* and *in vitro* to have an opposing effect on adipose tissue. GH causes the breakdown of triglycerides (lipolysis) in adipose tissue and in adipocyte cell culture models and induces release of glycerol and FFA (125). In addition, GH causes adipose tissue to be less sensitive to insulin stimulation. Mice deficient in GH are hypersensitive to insulin (127). Mice with excess circulating GH are insulin resistant while remaining lean and resistant to high fat diet-induced obesity (128). Human patients with GH excess, including acromegalics (chronic hyperpituitarism often

due to a tumor resulting in abnormally high GH secretion) have a high risk of developing insulin resistance and type II diabetes (129). Metabolic effects of GH in adipose tissue have been shown to be mediated, at least in part, by changes in gene expression (130-133). A mechanism by which GH is able to influence differential gene expression will be investigated in this report.

GH influences cell physiology by initiating a signal transduction cascade that originates by binding to a dimerized GH receptor (GHR):Janus Kinase 2 (JAK2) complex. One molecule of GH binds two molecules of the transmembrane GHR, which is thought to facilitate the placement of two molecules of JAK2 bound to GHR in an activated confirmation (29, 134, 135). JAK2 phosphorylates itself and GHR (29, 40, 136, 137). The resulting phospho-tyrosines serve as binding sites for downstream adaptor/scaffolding proteins, signal transducing kinases, and effector molecules (29, 138-140). This leads to the activation of multiple signaling pathways including IRS-PI3K-Akt (IRS interacts with the PH domain of SH2B1 and is recruited to active JAK2 where it is tyrosyl phosphorylated (110)), signal transducer and activator of transcription (STAT) proteins, and Extracellular Regulated Kinase 1/2 (ERK1/2) (29, 141, 142). The activation of these diverse pathways leads to further effector molecule modification/regulation and differential gene expression, enabling GH to exert its effect in a cell.

The adaptor protein SH2B1 is recruited to JAK2 in response to GH binding to GHR (22, 29). There are three members of the SH2B family: SH2B1 (SH2-B, PSM), SH2B2 (APS) and SH2B3 (Lnk) (26, 27, 29). All three family members

share the domain structure of a dimerization motif (N-terminal phenylalanine zipper), a pleckstrin homology domain, and an SH2 domain, as well as several proline-rich domains (reviewed in (29, 39)). While SH2B1 and SH2B2 are ubiquitously expressed in numerous tissues, SH2B3 is mainly expressed in hematopoietic tissues (143, 144). Four isoforms of SH2B1 (alpha, beta, gamma, and delta) produced by variable mRNA splicing have been identified. The four isoforms are identical in sequence except for the region downstream of the SH2 domain. Variable mRNA splicing confers unique C-terminal sequences for each isoform (28, 29). Recently, the four SH2B1 isoforms have been shown to contain a nuclear localization signal (NLS) and nuclear export sequence (NES), which have been shown to allow SH2B1β to cycle in and out of the nucleus (29, 66).

SH2B1 binds to phosphorylated tyrosine 813 on activated JAK2 via its SH2 domain and is tyrosyl phosphorylated by JAK2 (29, 40, 42). Over-expression of SH2B1 leads to enhanced tyrosyl phosphorylation and activity of JAK2. Enhanced JAK2 activation by over-expressed SH2B1 in COS cells expressing GHR has also been shown to lead to enhanced STAT5 tyrosyl phosphorylation and activation in response to GH (29, 108).

SH2B1 may be important for the metabolic regulation of adipose tissue, as it has recently been identified as a candidate obesity gene in humans (107) (112) and mice (18). SH2B1β has been implicated in the regulation of actin cytoskeleton dynamics (43, 45). Actin cytoskeletal rearrangements are a necessary prerequisite for cellular proliferation, differentiation and migration. In PC12 neuronal cells, SH2B1β has been shown to bind to and activate TrkA, the

receptor for nerve growth factor (NGF) (31), and promote NGF-induced neuronal differentiation in part by enhancing the expression of a sub-set of NGF induced genes required for neuronal differentiation (67). The NLS of SH2B1β allows it to traffic through the nucleus, and this nuclear-shuttling of SH2B1β is required for the enhancement of NGF-induced gene expression and differentiation (62). Thus, SH2B1 plays a role in the nucleus to regulate differential gene expression to mediate the cellular effects of NGF in PC12 cells, including neurite outgrowths.

3T3-F442A fibroblasts are a murine pre-adipocyte model cell line, which are able to differentiate into mature adipocytes that are highly sensitive to GH and have been used to characterize the metabolic effects of GH on adipocyte biology (145). SH2B1 has been shown to be required for GH-induced actin cytoskeleton rearrangement in 3T3-F442A pre-adipocytes. It interacts, directly or indirectly, with the small GTPase Rac, a known regulator of the actin cytoskeleton, and potentially Rac effector molecules in response to GH (29, 43, 45, 146). Because nuclear SH2B1 is important for NGF-induced gene expression in PC12 cells, there may be additional methods by which SH2B1 acts to mediate the effects GH, including direct regulation of gene transcription in the nucleus. In this report, we investigate the effects of SH2B1 on the regulation of GH-induced gene expression in 3T3-F442A adipocytes, with a particular emphasis on the nuclear role of SH2B1.

Methods and Materials

Antibodies and reagents. Polyclonal antibody to SH2B1 (1:1000) (110) was a kind gift from Dr. Liangyou Rui (University of Michigan, Ann Arbor, MI). Tubulin antibody (1:1000) is from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). IRDye800-conjugated anti-GFP, IRDye700 and IRDye800 conjugated anti-mouse and anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) were used at a dilution of 1:20,000. Recombinant 22,000-Da human GH was a kind gift from Eli Lilly & Co. (Indianapolis, IN).

Plasmids. Rat *SH2B1β* (NM_001048180) was cloned into the pEGFP C1 vector (Clontech, Mountain View, CA) (described previously (62)). Mutations were introduced using the QuickChange Mutagenesis kit (Stratagene) and have been described previously (62). To knock-down expression of SH2B1, the sequence targeting SH2B1: 5'-CATCTGTGGTTCCAGTCCA-3' was annealed and subcloned into pSuperior.retro.puro (Oligoengine, Seattle, WA) at *Bgl*II and *Xho*I sites. A control sequence of 5'-UUCUCCGAACGUGUCACGU-3' with no known target (QIAGEN- Xeragon, Germantown, MD) was also cloned into the same vector.

Cell culture and cell lines. The stock of murine 3T3-F442A pre-adipocytes was kindly provided by H. Green (Harvard University, Cambridge, MA). 3T3-F442A fibroblasts were stably transfected by retroviral infection. Retroviral infection was performed according to Erickson *et al.* (147). In brief, the recombinant plasmids were transfected into HEK 293T cells by calcium phosphate co-precipitation together with the viral packaging vectors SV-E-MLV-env and SVψ-E-MLV (148). Virus-containing medium was collected 16 h 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 sub-confluent (~30%) 3T3-F442A cells. The infection protocol was repeated twice with intervals of 16 hours. When cells achieved approximately 80% confluence, they were trypsinized, and cells expressing SH2B1 shRNA were stably selected in medium containing 2 μg/ml puromycin.

Microarray. RNA from murine 3T3-F442A stable cell lines was hybridized to Affymetrix Mouse Genome 430 2.0 Array gene chips. Hybridization and analysis of microarray results were conducted in conjunction with the University of Michigan Comprehensive Cancer Center's Affymetrix and cDNA Microarray Core Facility with supplemental funding provided by the Michigan Diabetes Training and Research Center (grant numbers 5P60 DK20572 & P30-CA46592).

Cell lysis and immunoblotting. Cells were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample lysis buffer (50 mM Tris [pH 7.5], 1% SDS, 0.001% bromophenol blue, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA) and proteins were separated by SDS-PAGE.

Proteins in gels were transferred to a nitrocellulose membrane and detected by immunoblotting with the indicated antibodies. Blots were then incubated with infrared dye-conjugated secondary antibodies and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

qPCR. Total RNA was isolated using Stat60 (Tel-Test) and cDNA was generated using TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). Gene expression was assessed using SYBR green I and the iCycler system with iCycler iQ Real Time Detection System software (Bio-Rad). Amplicons generated from each primer pair were 50–52 bp. The PCR well volume of each sample was normalized with fluorescein. All readings were normalized to the expression of GAPDH.

Results

Microarray analysis. Previously, SH2B1β has been seen to enhance the expression of a sub-set of NGF-induced genes in PC12 neuronal cells through microarray analysis (67). This sub-set of genes that have enhanced NGF-induced expression in cells overexpressing SH2B1β contains genes previously identified to be important for neuronal differentiation. Decreased gene expression was seen in response to NGF when SH2B1 was knocked down. We asked whether SH2B1 is having a similar effect on GH-induced gene expression in adipose cells. To gain insight into the function of SH2B1 in adipocytes, we carried out microarray analysis of GH-sensitive genes in 3T3-F442A adipocytes expressing a non-targeting control shRNA (3T3-F442A-shControl) or shRNA to SH2B1 (3T3-F442A-shSH2B1), before and after 1 h GH treatment (500 ng/ml) (FIG. 5.1). When the probe sets on the Affymetrix Mouse Genome 430 2.0 Array were re-mapped to represent only distinct genes, out of 16,361 total genes, 611 genes were up-regulated by GH by at least 60%. Of these 611 genes, the GH induction of 274 genes was reduced by at least 66% in 3T3- F442A-shSH2B1 cells vs. 3T3-F442A-shControl cells (FIG 5.2.). 120 genes fulfilled the more stringent criteria of being up-regulated by GH by at least 100%,

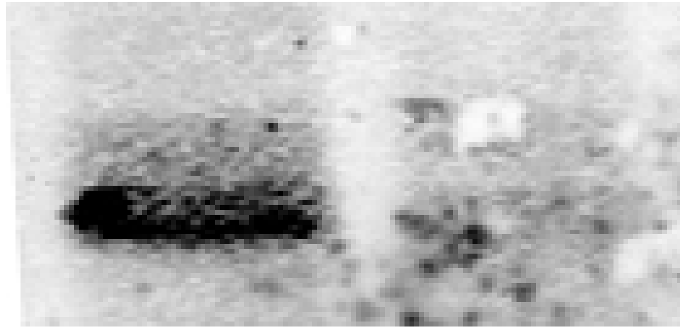


FIG. 5.1. SH2B1 expression knocked-down by shRNA. Stable expression of shRNA to SH2B1 (shSH2B1) in 3T3-F442A cells reduced SH2B1 levels by ~75% (compared to shControl cells). Immunoblotted with anti-SH2B1.

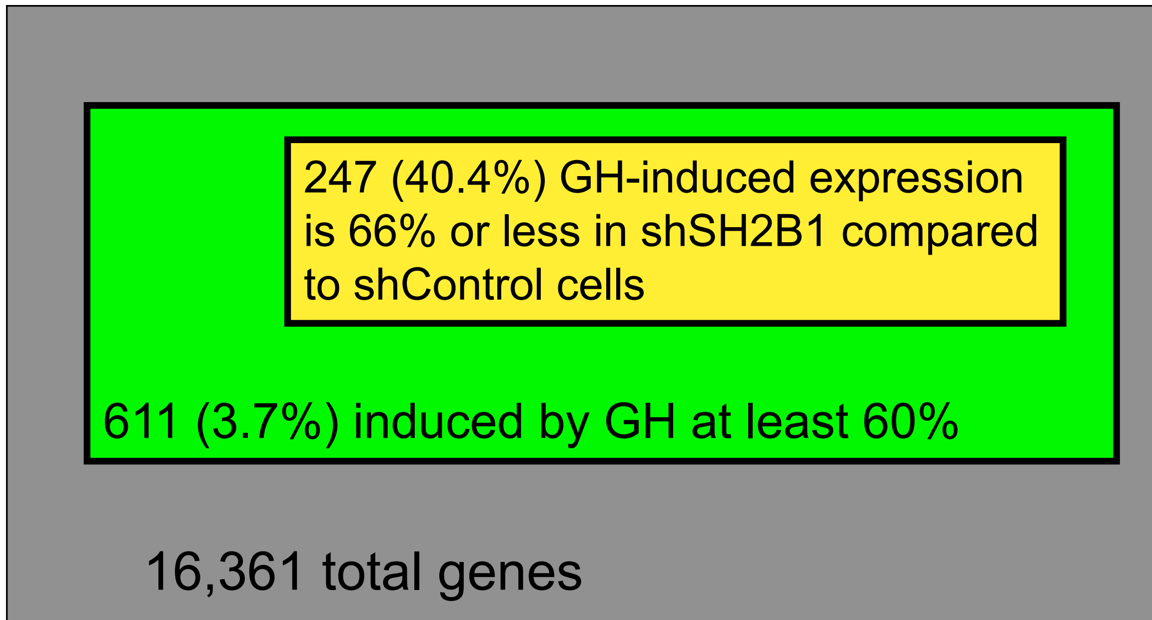


FIG. 5.2. Knock-down of SH2B1 decreases expression of 247 GH-dependent genes. 3T3-F442A cells stably expressing shControl or shSH2B1 were differentiated into adipocytes, serum-deprived overnight, and incubated with 500 ng/mL GH for 1h. mRNA was isolated and analyzed using Affymetrix Mouse Genome 430 2.0 chip.

with GH-induction reduced by at least 50% in 3T3-F442A-shSH2B1 cells compared to control 3T3-F442A-shControl cells.

Gene expression analysis by qPCR. To validate the results of the microarray, was performed to analyze expression levels of a select set of genes in 3T3-F442A cells in response to GH with or with SH2B1 knocked down. 3T3-F442A-shControl or 3T3-F442A-shSH2B1 cells were differentiated to adipocytes and treated with GH for 2 hours. Total RNA was then isolated and expression of the genes most highly up-regulated by GH identified in the microarray study was assessed by qPCR. In 3T3-F422A adipocytes, we have confirmed a corresponding ~50% reduction in GH-induced expression in the shSH2B1 cells for the genes *apelin* and *Cish* (FIG. 5.3). *Apelin* and *Cish* were highly expressed in response to GH in 3T3-F442A-shControl cells and this GH-induced expression was significantly reduced in 3T3-F442A-shSH2B1. We were able to confirm the GH-stimulated, but not the SH2B1-dependent expression of several other genes from the microarray study.

The gene *Arid5a* was identified through the microarray as being up-regulated by GH and dependent upon SH2B1 for its full GH-induced expression in adipocytes. However, *Arid5a* is more highly expressed in the pre-adipocyte state and detectable differences in gene expression were only seen from 3T3-F422A pre-adipocytes. Undifferentiated 3T3-F442A-shControl and 3T3-F442A-shSH2B1 cells were treated with GH for 1 hour and expression of *Arid5a* was analyzed by qPCR. *Arid5a* expression was up-regulated in response to GH in 3T3-F442A-shControl cells and this GH-induced expression was significantly

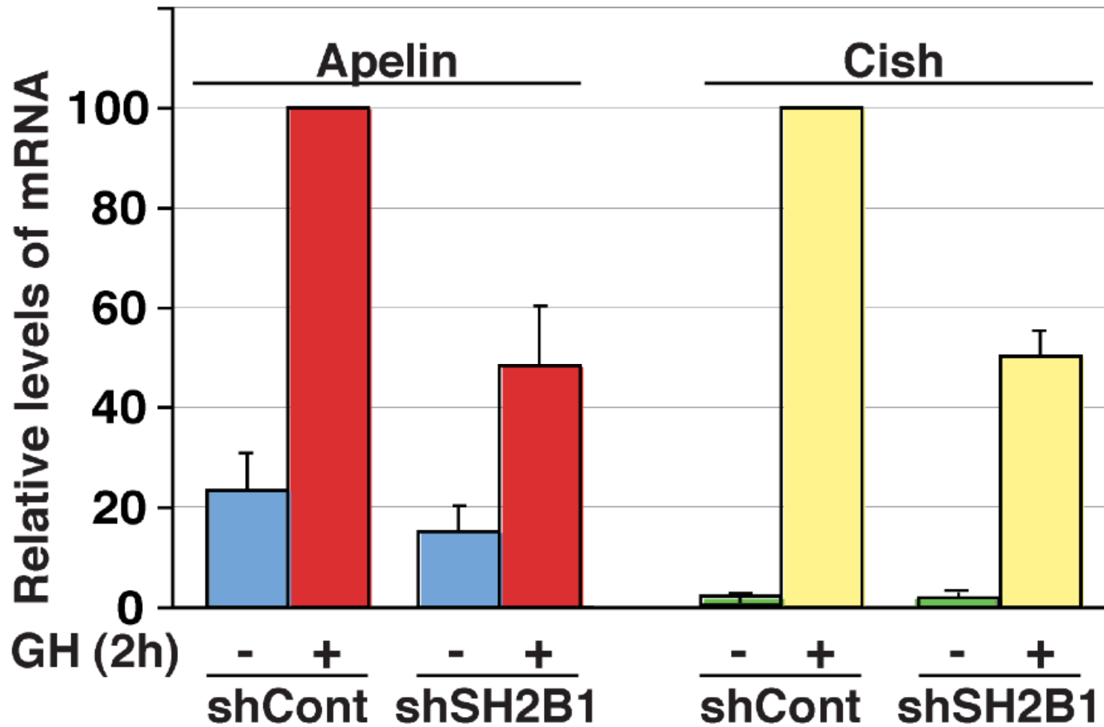


FIG. 5.3. Knock-down of SH2B1 decreases GH-dependent expression of Apelin and Cish. 3T3-F442A preadipocytes stably expressing shControl and shSH2B1 were differentiated into adipocytes, serum-deprived overnight and incubated with 500 ng/mL GH for 2h. mRNA levels were determined using qPCR, normalized to levels of GAPDH mRNA and then to the gene expression in GH-treated shControl cells. Means ± SEM (Apelin, n=3) or range (Cish, n=2) for different RNA preparations are shown.

decreased in 3T3-F442A-shSH2B1 cells. When SH2B1 WT was reintroduced into 3T3-F442A-shSH2B1 pre-adipocytes, full GH-induced expression of *arid5a* was rescued, with expression levels similar to those seen from 3T3-F442A-shControl cells (FIG. 5.4).

SH2B1 with a mutated NLS does not enhance GH-induced gene expression to the same extent as SH2B1 WT. To determine the role of nuclear SH2B1 in GH-induced gene expression, 3T3-F442A pre-adipocytes transiently expressing GFP, GFP-SH2B1β WT, or GFP-SH2B1β mNLS were stimulated with GH for 1 hour. Total RNA was isolated and gene expression was analyzed by qPCR. Cish expression in cells overexpressing SH2B1β WT was greatly enhanced over levels seen in control cells. However, Cish expression levels from cells overexpressing SH2B1β mNLS was not enhanced to the same extent as with WT (FIG. 5.5). Similar results were seen with *Apelin* and *Arid5a* expression (data not shown).

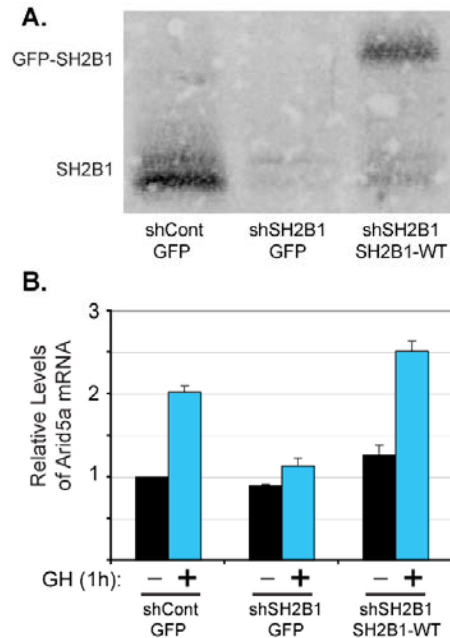


FIG. 5.4. Reintroduction of SH2B1β rescues GH-dependent induction of Arid5a mRNA in shSH2B1 expressing cells. (**A**) 3T3-F442A preadipocytes stably expressing shControl or shSH2B1 were rescued with GFP or GFP-SH2B1β rescue construct. Lysates were blotted with α-SH2B1. (**B**) These cell lines were serum-deprived overnight and incubated with 500 ng/mL GH for 1h. The GH-dependent induction of Arid5a mRNA was quantified using qPCR, normalized to levels of GAPDH mRNA and then to the gene expression in untreated shControl cells. Means and range of replicates are shown for a representative experiment.

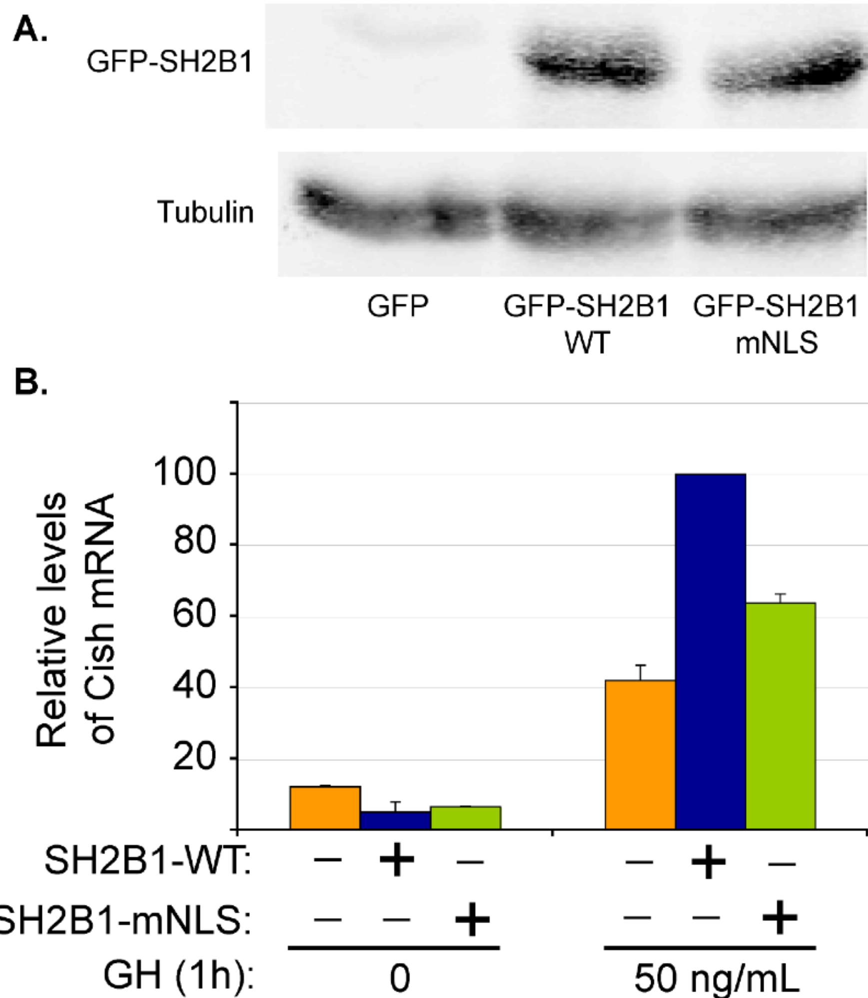


FIG. 5.5. The NLS in SH2B1β is required for maximal GH-induced expression of Cish mRNA. (**A**) 3T3-F442A preadipocytes overexpressing GFP, GFP-SH2B1β WT, or GFP-SH2B1β mNLS. Lysates were blotted with α-SH2B1 and α-Tubulin. (**B**) The above cell lines were serum- deprived overnight and incubated with 50 ng/mL GH for 1h. mRNA levels for Cish were determined using qPCR, normalized to levels of GAPDH mRNA and then to levels of mRNA in GH-treated GFP-SH2B1β−WT cells. Means and range of replicates are shown for a representative experiment.

Discussion

It has been shown previously that SH2B1β is important for regulating cellular functions of NGF and GH. SH2B1β with an intact NLS is capable of enhancing NGF-induced neuronal differentiation, in part by enhancing the expression of a sub-set of NGF-induced genes required for neuronal differentiation. We asked whether SH2B1 might play a similar role in adipose cells in response to GH. In this report, we elucidate the role of SH2B1β in GH-induced gene expression.

Through microarray analysis, we identified a sub-set of GH-induced genes that are dependent upon SH2B1β for their full GH-induced expression. We have confirmed by qPCR the SH2B1β-dependent GH-induced expression for three genes: apelin, arid5a, and Cish.

Apelin is a small protein hormone that is expressed with its receptor, APJ, in adipose tissue, brain, vascular endothelium, heart, lung, and kidney (149). Apelin is an adipokine that inhibits insulin secretion in mice, consistent with the anti-insulin effects of GH. Apelin promotes angiogenesis (150), which is important for the development and hypertrophy of fat depots. Its expression is strongly correlated with fat mass in mouse models and human patients, and

interestingly, is regulated by GH, IFN-γ, and insulin (151-154), three ligands thought to utilize SH2B1 as a signaling protein (22, 32, 33).

Arid5a (MRF1) is a member of the ARID (AT-rich interaction domain) family of DNA binding proteins, and was first identified as a repressor of the hCMV enhancer element, where it binds to its upstream, A+T rich modulator sequence (155). Recently, Arid5a was shown to interact with the transcription factor Sox9 to stimulate chondrocyte differentiation (156). Other ARID family member proteins have been shown to be important in regulating growth, differentiation, and development (157). The highly homologous Arid5b is important for proper growth and development of mice. Consistent with Arid5b playing a role in GH signaling, *Arid5b*$^{-/-}$ mice exhibit growth retardation, as well as reduced viability and abnormal development of reproductive organs (158). *Arid5b*$^{-/-}$ mice were also lean, with significant reductions in brown and white adipose tissues, and resistant to weight gain and obesity when maintained on a high-fat diet (159). This suggests that Arid5b is important for accumulating lipid stores and adipogenesis. Arid5a is ubiquitously expressed with the highest expression levels in skeletal muscle, liver and heart, is able to interact with several nuclear hormone receptors, and can specifically repress the transcriptional activity of estrogen receptors α/β and the androgen receptor (160).

Cytokine-inducible SH2 protein (Cish) is a member of the suppressors of cytokine signaling (SOCS) family whose expression is highly induced by a range of cytokines, including GH, and is ubiquitously expressed (161-163). The promoter region of Cish contains a STAT-binding sequence and its expression is

induced by activation of the JAK-STAT pathway (164). Cish functions in a classical negative feedback loop to inhibit JAK-STAT signaling (162, 165).

We also showed that SH2B1β with a mutant NLS is not capable of enhancing GH-induced gene expression to the same extent as SH2B1β WT, which suggests that nuclear shuttling of SH2B1 is important for the regulation of GH-induced gene expression. However, while these results were intended to determine whether nuclear shuttling of SH2B1β is required for the SH2B1β regulation of a sub-set of GH-induced genes, it was later realized that not only does mutating the NLS of SH2B1β prevent it from entering the nucleus, SH2B1β mNLS also does not localize the PM as the NLS of SH2B1β is a dual localization motif (65). Thus, it may only be that PM localization of SH2B1β (where it interacts with GHR/JAK2 to enhance JAK2 activity) is what is required for SH2B1β to promote enhanced expression of GH-induced genes. Nevertheless, we can conclude that an intact NLS, which ensures both PM and nuclear localization of SH2B1β, is required for the full GH-induced expression of SH2B1-dependent genes.

Chapter 6

Conclusion

Summary and Future Directions

The overall goal of my thesis research was to elucidate functions of different SH2B1 isoforms (particularly alpha, beta, and delta). One approach took advantage of human mutations identified in patients with severe obesity, insulin resistance, and maladaptive behavior. Our lab was particularly interested to understand if human mutations in *SH2B1* identified in patients with severe obesity, insulin resistance, and maladaptive behavior lead to dysfunction of SH2B1 protein and how, mechanistically, the human mutation conferred dysfunction on SH2B1. There are four isoforms of SH2B1 that mostly behave similar to each other (28), yet there are instances when an isoform displays unique properties. The beta and gamma isoforms are of similar length, typically expressed together (112), and have been seen to behave very similarly in cellular assays (49). The alpha and delta isoforms are longer transcripts and would include additional human mutations that would not be expressed in the shorter beta and gamma transcripts. However, our lab has seen major differences in sub-cellular distribution for these isoforms suggesting there may be substantial differences in their function. Alpha is at the plasma membrane (PM) and cytosol but not detected in the nucleus even in the presence of a nuclear

export inhibitor. Beta and gamma have been seen to be present in the PM and cytosol and accumulate in the nucleus in the presence of a nuclear export inhibitor. In contrast, delta has a steady-state sub-cellular localization at the PM and at the nucleolus. Thus, we must elucidate the specific cellular functions of the separate SH2B1 isoforms in order to understand how the cellular functions of specific isoforms are affected by human mutations identified in *SH2B1*. The effects of the human mutations on the cellular functions of SH2B1 alpha, beta, and delta are investigated in this report, with particular attention paid to the effects on NGF-induced neuronal differentiation.

Mutations in *SH2B1* were identified in patients with hyperphagic obesity, extreme insulin resistance, and maladaptive behavior. The phenotype of the human patients closely resembles that of the SH2B1-KO mouse where systemic leptin resistance is thought to be a primary factor leading to obesity (18). Due to the fact that the phenotype of SH2B1-KO mouse can be ameliorated with transgenic expression of SH2B1 mainly in neurons (54) and that SH2B1 is important for neuronal differentiation and survival (30, 31), we investigated the effects of the human mutations on the function of SH2B1β in a neuronal context. The human and rodent phenotypes would suggest that mutant SH2B1 is disrupting leptin signaling. However, no difference in the ability to promote JAK2 activation or leptin-stimulated (and insulin-stimulated) IRS2 tyrosyl phosphorylation was detected between WT and mutant SH2B1β. We did detect impairment, however, in the ability of mutant SH2B1β to enhance NGF-induced neurite outgrowth and GH-induced macrophage migration compared to SH2B1β

WT. While signaling at the level of the plasma membrane does not appear to be affected by the human mutations, proper migration and differentiation of neuronal precursor cells may be impaired in patients with mutations in *SH2B1*. However, our assays relied on overexpression of leptin receptor, IRS2 and SH2B1 in 293T cells that may have masked any subtle differences in leptin-induced IRS2 tyrosyl phosphorylation. The human mutations may also be affecting other actions of leptin that were not measured in this report. The effect of SH2B1 with human mutations on leptin signaling should be investigated in primary neurons that express endogenously the leptin receptor. It may be that leptin neuro-endocrine signaling is still disrupted by improper development or maintenance of neurons necessary for mediating the effects of leptin and improper neuronal development may also explain the cognitive and behavior abnormalities associated with the human mutations in *SH2B1*.

In order to elucidate the role of SH2B1 in neuronal development, SH2B1-KO mice will be made that transgenically express WT or mutant SH2B1β in neurons in an inducible manner. I would expect that mice that express transgenic neuronal SH2B1β from inception would not develop an obese phenotype. I would expect transgenic mutant SH2B1β expressed at any time point before or subsequent to birth, to not be able to protect against the obese phenotype. A finding that postnatally expressed SH2B1β is able to rescue the obese phenotype would suggest that SH2B1 is not required from inception for proper neural development and raises the question of whether impaired leptin signaling is the primary factor causing hyperphagia in SH2B1-KO mice. SH2B1-

KO mice do not respond to exogenous leptin administration by reducing food intake and body weight (18), implying that leptin signaling in leptin receptor-expressing neurons is severely disrupted. This would also suggest that therapeutic treatment that increases the activity of leptin downstream of SH2B1 in human patients, like MC4R agonists or inhibitors of NPY or AgRP function, might be a viable approach to treat obesity in the patients with mutations in *SH2B1*. Expressing the alpha, gamma, and delta isoforms in the animals similarly to SH2B1β would help elucidate whether the different SH2B1 isoforms have overlapping and/or unique functions during neuronal development.

The neuronal assays utilized throughout this report focus on the effect of SH2B1 on NGF signaling in PC12 cells. SH2B1 has also been shown to interact with the activated receptors for BDNF and GDNF (30, 35, 64). Investigating the role of SH2B1 in BDNF signaling is particularly relevant for these studies because genetic variations that cause reduced BDNF or TrkB expression or activity lead to hyperphagia and obesity in humans and mice (13, 14, 166-169). To support a role for SH2B1 in neuronal development, neuronal signaling and development assays using BDNF or GDNF and cortical precursor cells (or other primary neuronal tissues) will be conducted. Dysfunction in these assays with mutant SH2B1 would broaden the scope of where SH2B1 with human mutations are having an effect on neuronal function from just NGF signaling.

Although SH2B1α has been reported to behave similarly to SH2B1β in promoting mitogenesis and insulin receptor activity (28, 49), we did not see SH2B1α enhance NGF-induced PC12 cell neuronal differentiation as we saw

with SH2B1β. Obese patients with mutations that would only be expressed in the alpha isoform do not exhibit the maladaptive behavior phenotype. Preliminary results also suggest that human mutations unique to SH2B1α do not affect GH-induced macrophage motility (44). This evidence would suggest that SH2B1α does not play an important role in neuronal development, yet patients with mutations in the alpha isoform are still obese and insulin resistant. SH2B1α was shown to enhance insulin receptor activity to a greater extent than other SH2B1 isoforms (49). We showed that insulin-stimulated IRS2 tyrosyl phosphorylation is not affected by human mutations in SH2B1α, yet the nature of this assay with the overexpression of SH2B1α and IRS2 may mask any subtle differences in phosphorylation. Human mutations in SH2B1α may be affecting other actions of insulin we did not measure in this report.

If insulin signaling is in fact affected by human mutations in SH2B1α, we may be able to see differences in assays measuring other responses to insulin. Insulin-sensitive cells, such as 3T3-L1 adipocytes, will be transfected with cDNA encoding for WT or mutant SH2B1α, treated with a low concentration of insulin (0.1-10 nM), and assayed for glucose uptake, amino acid transport, glycogen synthesis, and lipogenesis. If a dysfunction is observed in any of these assays with mutant SH2B1α versus WT, the signaling molecules important for the regulation of this response to insulin will be investigated. I would expect that the IRS-PI3K pathway would not be affected based on previous results, however that does not exclude downstream effectors or parallel signaling pathways from being affected.

While insulin-signaling potentiation may be an important role for SH2B1α, it would still appear that SH2B1 activity in the brain is sufficient to maintain whole-body energy homeostasis (54). To further elucidate a neuronal role for SH2B1α, it would be key to first learn where in the brain SH2B1α is being expressed. Based on the specific tissues or cell types identified, specific assays to test WT or mutant SH2B1α function will be employed. One example assay could be to measure conductance in nerve tissue where SH2B1α would be normally expressed between WT and mutant SH2B1α. SH2B1α maybe affecting ion channel or synapse function.

I showed that SH2B1δ localized to the nucleus/nucleolus through its C-terminal bipartite NLS and that this localization is important for SH2B1δ to enhance NGF-induced neuronal differentiation. However, I was unable to identify a mechanism by which human mutations in SH2B1δ impair the ability of SH2B1δ to enhance neuronal differentiation. Identifying binding partners of SH2B1δ in the nucleus/nucleolus and how the human mutations affect the ability of SH2B1δ to enhance neuronal differentiation will further elucidate the role of SH2B1 in neuronal development.

We did not clearly identify the region of the SH2B1δ that is responsible for targeting SH2B1δ to the nucleolus. Our combination of nuclear localization signal (NLS) mutations in SH2B1δ did not result in a nuclear but non-nucleolar sub-cellular localization of SH2B1δ for any construct tested. For example, SH2B1δ mNLS2 and mNLS3 were present in the nucleolus, however SH2B1δ mNLS2/3 was not present in the nucleus at all. This suggests that while NLS 2

and NLS 3 are responsible for nuclear entry of SH2B1δ and could be targeting it to the nucleolus, another region may be directing SH2B1δ to localize specifically at the nucleolus. RNA-binding motifs (stretches of aromatic amino acids) and glycine/arginine-rich (GR) domains have been implicated in targeting nuclear proteins to the nucleolus (124). The SH2B1δ C-terminal tail contains a Trp-rich region as well as GR segments within the bipartite NLS (28). A series of deletion mutants as well as mutations targeting GR segments or the Trp-rich region will be used to identify the C-terminal region of SH2B1δ responsible for nucleolar localization. Once this region is identified, forms of SH2B1δ that allow for nuclear but not nucleolar localization will be assayed for their capacity to promote NGF-induced neuronal differentiation.

I would expect WT but not nucleolar-excluded SH2B1δ to be able to bind RNA and other nucleolar or ribosomal proteins. It would be interesting to compare a global screen of binding partners in response to NGF between SH2B1δ WT, non-nuclear SH2B1δ, non-nucleolar SH2B1δ, and SH2B1β. I would expect that I would identify many proteins common to SH2B1δ and SH2B1β. However, differences between interacting partners of SH2B1δ WT and non-nuclear/nucleolar SH2B1δ (that presumably would not enhance NGF-induced neuronal differentiation) may provide great insight into what SH2B1δ might be doing in the nucleolus. Interestingly, when SH2B1β accumulates in the nucleus, it appears excluded from nucleoli (112). This difference in sub-nuclear localization may suggest that the beta and delta SH2B1 isoforms are acting to regulate neuronal differentiation by different mechanisms. Identifying differential

binding partners of the two isoforms may help elucidate these differing mechanisms.

There are a number of proteins in the nucleus that regulate neurogenesis that may be affected by mutant SH2B1 (170). Phosphorylation in response to NGF in cells expressing WT or mutant SH2B1δ will be investigated for proteins such as Elk-1, serum response factor (SRF), cAMP response element-binding protein (CREB), and ribosomal S6 kinase (RSK). I would expect phosphorylation of one or more of these proteins essential for neuronal differentiation would be impaired from cells expressing mutant SH2B1δ compared to WT.

It may be that SH2B1δ acts to increase ribosome biosynthesis from nucleoli to promote neuritogenesis. Activation of RNA polymerase I (Pol1), which transcribes ribosomal DNA, is sufficient to cause cellular hypertrophy and neurite outgrowth in neuronal cells (reviewed in (123)). Pol1 is activated by transcription initiation factor-1A (TIF1A) and TIF1A is activated by phosphorylation on serine residues 633 and 649 by ERK 2 and RSK2 (which is activated by ERK 1/2), respectively (171). We have shown that SH2B1δ enhances ERK 1/2 phosphorylation in response to NGF. Thus, I hypothesize that TIF1A will be more highly phosphorylated in response to NGF from cells expressing SH2B1δ WT resulting in increased differentiation compared SH2B1δ with a human mutation. A finding that signaling through TIF1A is not reduced when a human mutation is introduced into SH2B1δ, would suggest that impairment of neurite outgrowth in response to NGF seen when PC12 cells express SH2B1δ with a human mutation is not due to impaired regulation of ribosome biosynthesis.

Through microarray analysis, we were able to identify a subset of genes that required SH2B1 for their full GH-induced expression. We also showed that an intact NLS motif (which targets SH2B1β to the plasma membrane and nucleus (65)) in SH2B1β is required for SH2B1β to maximally enhance a sub-set of GH-induced genes. However, we were unable to conduct the experiments to elucidate a mechanism by which SH2B1β regulates gene expression due to time constraints.

I hypothesize that SH2B1β interacts with transcription factors to assemble transcription-activating complexes, or acts to shuttle transcription factors that repress transcription out of the nucleus to then allow for activation of transcription. Gene Set Enrichment Analysis from the set of SH2B1-dependent, GH-induced genes identified a list of transcription factor binding motifs that are statistically over-represented in this set (Table 6.1). Interaction of these transcription factors with SH2B1β in response to GH will be assayed to gain understanding of how SH2B1β acts in the nucleus to regulate gene expression. I would expect SH2B1β WT, but not SH2B1β mNLS, to interact with one or more of these transcription factors to regulate gene transcription.

I used proteomics with mass spectrometry to identify binding proteins of Flag-tagged SH2B1β in response to GH (Table 6.2). Confirmation of SH2B1β interaction with proteins will be prioritized based on relevance. I would prioritize assaying SH2B1β interaction with signaling proteins Map3k7 and Tab3 (a binding partner of Map3k7), as well as RNA-handling proteins like Adar and Sfrs 2/3. The MAP kinase signaling pathway is an important regulator of gene transcription

Table 6.1. Identification of over-represented trancription factor binding site sequence motifs from gene set enrichment analysis of GH-induced, SH2B1-dependent genes.

Transcription Factor	Sequence Motif	Total genes	Hot genes
NRF1	CGCATGCGCR	155	12
NRF1	RCGCANGCGY	566	29
YY1	GCCATNTTG	279	21
YY1	NNNCGGCCAT CTTGNCTSNW	156	11
E2F	NCSCGCSAAAN	161	12
E2F	TTTCGCGC	154	11
OCT1	NNNRTAATNANNN	159	12
PAX3	CGTSACG	102	9
FOXO3	TNNTTGTTTACNTW	166	12
HNF1	DGTTAATKAW TNACCAM	166	12
C/EBP	NTTRCNNAANNN	194	13
SRF	SCCAWATAWG GMNMNNNN	174	12
IRF	BNCRSTTTCANTTYY	155	11

From Gene Set Enrichment Analysis

Over-represented transcription factor binding sequence motifs with p-value < 0.01.
N = any nt, B = not A, D = not C, H = not G, V = not T, K = G/T, Y = C/T, S = C/G, W = A/T, R = A/G, M = A/C

and neuronal differentiation. SH2B1δ localizes at the nucleolus which is a major site of RNA processing and several RNA-handling proteins were identified in our screen. The regulation of RNA processing is another mechanism by which SH2B1 may be regulating gene expression.

Carrying out these experiments will provide important insight into how the different SH2B1 isoforms act to regulate cytokine and growth factor signaling and how they are affected by human mutations identified in *SH2B1*. Further understanding of the mechanism of action of SH2B1 may provide basis for therapeutic intervention to treat obesity and/or insulin resistance in humans.

A schematic summarizing the functions of SH2B1 in response to neurotrophic factors and how they are impaired by the human mutations is depicted in FIG. 6.1.

Table 6.2. Identification of potential interacting partner of SH2B1. Flag-SH2B1β was immunoprecipitated from 3T3-F442A adipocytes after stimulation with GH (500 ng/ml) for 1 hour. Proteins were detected by mass spectrometry and an abbreviated list of relevant proteins are presented.

Proteins identified as potential SH2B1-interacting proteins
Adar (Adenosine deaminases acting on RNA)
Hdx (Isoform 1 of Highly divergent homeobox)
Pcbp1 (Poly(rC)-binding protein 1)
Prpf31 (Isoform 1 of U4/U6 small nuclear ribonucleoprotein)
Setx (Isoform 1 of Probable helicase senataxin)
Sfrs2 (Splicing factor, arginine/serine-rich 2)
Sfrs3 (Isoform Long of Splicing factor, arginine/serine-rich)
Smad1 (TGF-beta-signaling transcription factor)
Sub1 (Activated RNA polymerase II transcriptional coactivator)
Thrap3 (Thyroid hormone receptor-associated protein 3)
Map3k7 (Mitogen-activated protein kinase kinase kinase 7)
Tab3 (TGF-beta-activated kinase 1 and MAP3K7-binding protein)
Grid2ip (Glutamate receptor, ionotropic, delta 2-interacting protein 1)

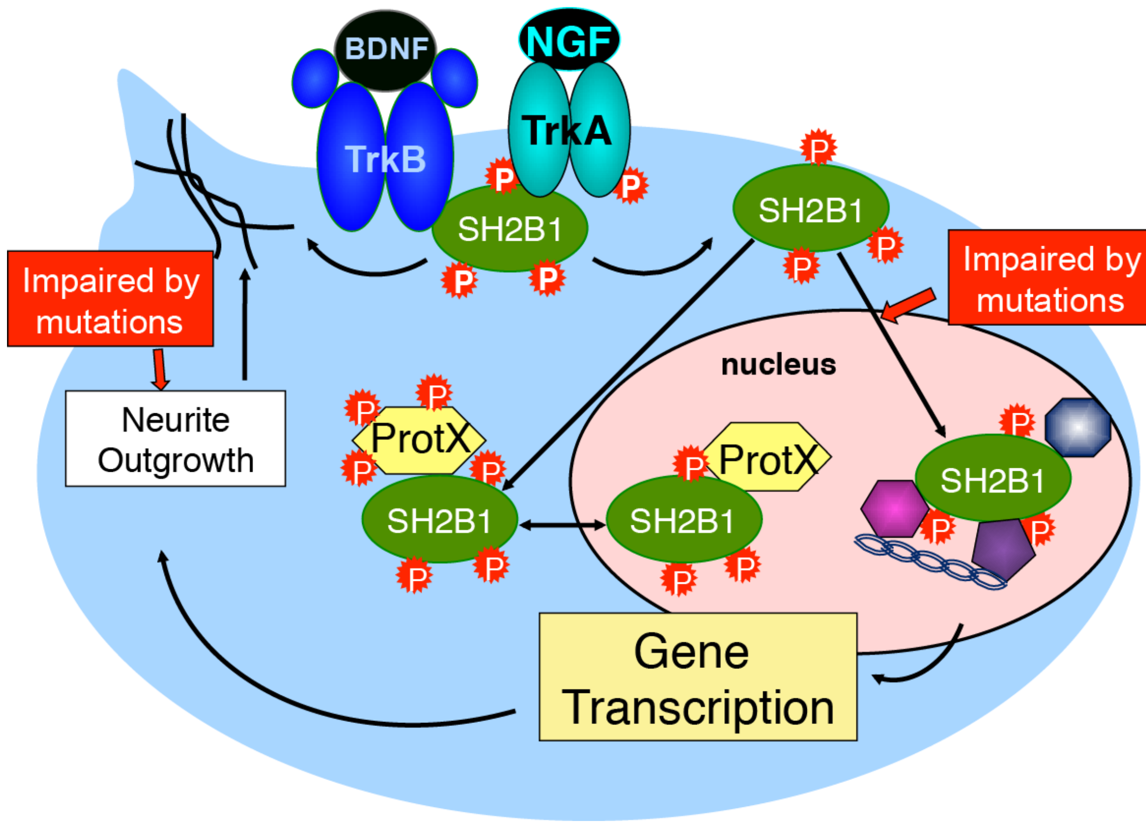


FIG. 6.1. Schematic of SH2B1 function in response to neurotrophic factors and where it is impaired by human mutations. SH2B1 localizes at the plasma membrane and cytosol and cycles in and out of the nucleus. SH2B1 acts to enhance NGF-induced gene expression and neurite outgrowth. It is hypothesized that SH2B1 acts to form a transcription-activating complex in the nucleus or promotes gene transcription by shuttling a repressor transcription factor out of the nucleus or a signaling molecule into the nucleus (ProtX). SH2B1 translocation to the nucleus and ability to enhance neurite outgrowth in response to neurotrophic factors are impaired by human mutations identified in SH2B1.

Table 6.3

cDNA	Assay										
	JAK2 activation	Lep stim. pIRS2	Ins stim. pIRS2	PM localization	Nucleolar localization	Nuclear Accumulation	PC12 differentiation	Macrophage migration	NGF-stim pERK1/2	NGF-stim gene expression	Dimerization
SH2B1β WT	Y	Y	Y	Y		Y	Y	Y	N	Y	Y
SH2B1β P90H	Y	Y	Y	Y		Impaired	Impaired	N			Y
SH2B1β T175N	Y	Y	Y	Y		Impaired	Impaired	N			Y
SH2B1β P322S	Y	Y	Y	Y		Impaired	Impaired	N			Y
SH2B1β F344LfsX20	N			Impaired		Impaired	N	N			Y
SH2B1α WT	Y		Y	less than β		N	N			N	
SH2B1α P90H	Y		Y	less than β							
SH2B1α T175N	Y		Y	less than β							
SH2B1α G238C	Y		Y	less than β							
SH2B1α R270W	Y		Y	less than β							
SH2B1α P322S	Y		Y	less than β							
SH2B1α F344LfsX20	N		N	less than β							
SH2B1α A484T	Y		Y	less than β							
SH2B1α T564A	Y		Y	less than β							
SH2B1α A663V	Y		Y	less than β							
SH2B1α V695M	Y		Y	less than β							
SH2B1α A723V	Y		Y	less than β							
SH2B1δ WT	Y			Y	Y		Y		Y	Y	
SH2B1δ P90H	Y						Impaired				
SH2B1δ T175N	Y						Impaired				
SH2B1δ P322S	Y						Impaired		Y	Y	
SH2B1δ A484T	Y						Y				
SH2B1δ T564A	Y						Impaired				
SH2B1δ R680C	Y						Impaired		Y	Y	
SH2B1δ P712P	Y						Y				

Table 6.3. Summary of assays presented in this dissertation by isoform of SH2B1. Assays are listed across the top with SH2B1 cDNA used in the assay in left column. Y = cDNA used enhanced assay, N = cDNA used did not enhance assay.

References

1. **Ogden CL, Carroll MD, Kit BK, Flegal KM** 2012 Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. Jama 307:483-490

2. **Farooqi S, O'Rahilly S** 2006 Genetics of obesity in humans. Endocrine Rev 27:710-718

3. **Farooqi IS, O'Rahilly S** 2005 Monogenic obesity in humans. Annu Rev Med 56:443-458

4. **Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG** 1996 Identification of targets of leptin action in rat hypothalamus. J Clin Invest 98:1101-1106

5. **Morris DL, Rui L** 2009 Recent advances in understanding leptin signaling and leptin resistance. Am J Physiol Endocrinol Metab 297:E1247-E1259

6. **Friedman JM, Halaas JL** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763-770

7. **Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S** 2002 Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest 110:1093-1103

8. **Gibson WT, Farooqi IS, Moreau M, DePaoli AM, Lawrence E, O'Rahilly S, Trussell RA** 2004 Congenital leptin deficiency due to homozygosity for the Δ133G mutation: report of another case and evaluation of response to four years of leptin therapy. J Clin Endo Metab 89:4821-4826

9. **Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD** 1998 A leptin missense mutation associated with hypogonadism and morbid obesity. Nature Genet 18:213-215

10. **Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B** 1998 A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392:398-401

11. **Schwartz MW, Woods SC, Porte Jr. D, Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661-671

12. **Coll AP, Farooqi IS, Challis BG, Yeo GS, O'Rahilly S** 2004 Proopiomelanocortin and energy balance: insights from human and murine genetics. J Clin Endo Metab 89:2557-2562

13. **Yeo GS, Connie Hung CC, Rochford J, Keogh J, Gray J, Sivaramakrishnan S, O'Rahilly S, Farooqi IS** 2004 A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nat Neurosci 7:1187-1189

14. **Gray J, Yeo GS, Cox JJ, Morton J, Adlam AL, Keogh JM, Yanovski JA, El Gharbawy A, Han JC, Tung YC, Hodges JR, Raymond FL, O'Rahilly S, Farooqi IS** 2006 Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. Diabetes 55:3366-3371

15. **Han JC, Liu QR, Jones M, Levinn RL, Menzie CM, Jefferson-George KS, Adler-Wailes DC, Sanford EL, Lacbawan FL, Uhl GR, Rennert OM, Yanovski JA** 2008 Brain-derived neurotrophic factor and obesity in the WAGR syndrome. N Engl J Med 359:918-927

16. **Huang EJ, Reichardt LF** 2001 Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677-736

17. **Liao GY, An JJ, Gharami K, Waterhouse EG, Vanevski F, Jones KR, Xu B** 2012 Dendritically targeted *Bdnf* mRNA is essential for energy balance and response to leptin. Nature Med 18:564-571

18. **Ren D, Li M, Duan C, Rui L** 2005 Identification of SH2-B as a key regulator of leptin sensitivity, energy balance and body weight in mice. Cell Metab 2:95-104

19. **Li Z, Zhou Y, Carter-Su C, Myers Jr. MG, Rui L** 2007 SH2B1 enhances leptin signaling by both Janus kinase 2 Tyr^{813} phosphorylation-dependent and -independent mechanisms. Mol Endocrinol 21:2270-2281

20. **Ghilardi N, Skoda RC** 1997 The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. Mol Endocrinology 11:393-399

21. **Banks AS, Davis SM, Bates SH, Myers Jr. MG** 2000 Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275:14563-14572

22. **Rui L, Mathews LS, Hotta K, Gustafson TA, Carter-Su C** 1997 Identification of SH2-Bβ as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling. Mol Cell Biol 17:6633-6644

23. **O'Brien KB, O'Shea JJ, Carter-Su C** 2002 SH2-B family members differentially regulate JAK family tyrosine kinases. J Biol Chem 277:8673-8681

24. **Kuriyan J, Cowburn D** 1997 Modular peptide recognition domains in eukaryotic signaling. Ann Rev Biophys Biomol Struct 26:259-288

25. **Osborne MA, Dalton S, Kochan JP** 1995 The Yeast Tribrid System - Genetic Detection of *trans*-phosphorylated ITAM-SH2-Interactions. BioTechnology 13:1474-1478

26. **Yokouchi M, Suzuki R, Masuhara M, Komiya S, Inoue A, Yoshimura A** 1997 Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. Oncogene 15:7-15

27. **Huang X, Li Y, Tanaka K, Moore KG, Hayashi JI** 1995 Cloning and characterization of Lnk, a signal transduction protein that links T-cell receptor activation signal to phospholipase C g 1, Grb2, and phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 92:11618-11622

28. **Yousaf N, Deng Y, Kang Y, Riedel H** 2001 Four PSM/SH2-B alternative splice variants and their differential roles in mitogenesis. J Biol Chem 276:40940-40948

29. **Wells JA** 1996 Binding in the growth hormone receptor complex. Proc Natl Acad Sci USA 93:1-6

30. **Qian X, Riccio A, Zhang Y, Ginty DD** 1998 Identification and characterization of novel substrates of Trk receptors in developing neurons. Neuron 21:1017-1029

31. **Rui L, Herrington J, Carter-Su C** 1999 SH2-B is required for nerve growth factor-induced neuronal differentiation. J Biol Chem 274:10590-10594

32. **Riedel H, Wang J, Hansen H, Yousaf N** 1997 PSM, an insulin-dependent, pro-rich, PH, SH2 domain containing partner of the insulin receptor. J Biochem 122:1105-1113

33. **Nelms K, O'Neill TJ, Li S, Hubbard SR, Gustafson TA, Paul WE** 1999 Alternative splicing, gene localization, and binding of SH2-B to the insulin receptor kinase domain. Mammalian Genome 10:1160-1167

34. **Wang J, Riedel H** 1998 Insulin-like growth factor-I receptor and insulin receptor association with a Src homology-2 domain-containing putative adapter. J Biol Chem 273:3136-3139

35. **Zhang Y, Zhu W, Wang YG, Liu XJ, Jiao L, Liu X, Zhang ZH, Lu CL, He C** 2006 Interaction of SH2-Bβ with RET is involved in signaling of GDNF-induced neurite outgrowth. J Cell Sci 119:1666-1676

36. **Rui L, Carter-Su C** 1998 Platelet-derived growth factor (PDGF) stimulates the association of SH2-Bβ with PDGF receptor and phosphorylation of SH2-Bβ. J Biol Chem 273:21239-21245

37. **Kong M, Wang CS, Donoghue DJ** 2002 Interaction of fibroblast growth factor receptor 3 and the adapter protein SH2-B. J Biol Chem 277:15962-15970

38. **Nishi M, Werner ED, Oh BC, Frantz JD, Dhe-Paganon S, Hansen L, Lee J, Shoelson SE** 2005 Kinase activation through dimerization by human SH2-B. Mol Cell Biol 25:2607-2621

39. **Maures TJ, Kurzer JH, Carter-Su C** 2007 SH2B1 (SH2-B) and JAK2: a multifunctional adaptor protein and kinase made for each other. Trends Endocrinol Metab 18:38-45

40. **Kurzer JH, Argetsinger LS, Zhou Y-J, Kouadio J-L, O'Shea JJ, Carter-Su C** 2004 Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-Bβ. Mol Cell Biol 24:4557-4570

41. **Saharinen P, Takaluoma K, Silvennoinen O** 2000 Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Mol Cell Biol 20:3387-3395

42. **O'Brien KB, Argetsinger LS, Diakonova M, Carter-Su C** 2003 YXXL motifs in SH2-Bβ are phosphorylated by JAK2, JAK1, and platelet-derived growth factor receptor and are required for membrane ruffling. J Biol Chem 278:11970-11978

43. **Herrington J, Diakonova M, Rui L, Gunter DR, Carter-Su C** 2000 SH2-B is required for growth hormone-induced actin reorganization. J Biol Chem 275:13126-13133

44. **Su HW, Lanning NJ, Morris DL, Argetsinger LS, Lumeng CN, Carter-Su C** 2012 Phosphorylation of the adaptor protein SH2B1β regulates its ability to enhance growth hormone (GH)-dependent macrophage motility.Submitted for publication

45. **Diakonova M, Gunter DR, Herrington J, Carter-Su C** 2002 SH2-Bβ is a Rac-binding protein that regulates cell motility. J Biol Chem 277:10669-10677

46. **Kotani K, Wilden P, Pillay TS** 1998 SH2-Bα is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase. Biochem J 335:103-109

47. **Hu J, Hubbard SR** 2006 Structural basis for phosphotyrosine recognition by the Src homology-2 domains of the adapter proteins SH2-B and APS. J Mol Biol 361:69-79

48. **Riedel H, Yousaf N, Zhao Y, Dai H, Deng Y, Wang J** 2000 PSM, a mediator of PDGF-BB-, IGF-I-, and insulin-stimulated mitogenesis. Oncogene 19:39-50

49. **Zhang M, Deng Y, Tandon R, Bai C, Riedel H** 2008 Essential role of PSM/SH2-B variants in insulin receptor catalytic activation and the resulting cellular responses. J Cell Biochem 103:162-181

50. **Ahmed Z, Pillay TS** 2003 Adapter protein with a pleckstrin homology (PH) and an Src homology 2 (SH2) domain (APS) and SH2-B enhance insulin-receptor autophosphorylation, extracellular-signal-regulated kinase and phosphoinositide 3-kinase-dependent signalling. Biochem J 371:405-412

51. **Morris DL, Cho KW, Zhou Y, Rui L** 2009 SH2B1 enhances insulin sensitivity by both stimulating the insulin receptor and inhibiting tyrosine dephosphorylation of insulin receptor substrate proteins. Diabetes 58:2039-2047

52. **Duan C, Yang H, White MF, Rui L** 2004 Disruption of SH2-B causes age-dependent insulin resistance and glucose intolerance. Mol Cell Biol 24:7435-7443

53. **Li M, Ren D, Iseki M, Takaki S, Rui L** 2006 Differential role of SH2-B and APS in regulating energy and glucose homeostasis. Endocrinology 147:2163-2170

54. **Ren D, Zhou Y, Morris D, Li M, Li Z, Rui L** 2007 Neuronal SH2B1 is essential for controlling energy and glucose homeostasis. J Clin Invest 117:397-406

55. **Ohtsuka S, Takaki S, Iseki M, Miyoshi K, Nakagata N, Kataoka Y, Yoshida N, Takatsu K, Yoshimura A** 2002 SH2-B is required for both male and female reproduction. Mol Cell Biol 22:3066-3077

56. **Yoshiga D, Sato N, Torisu T, Mori H, Yoshida R, Nakamura S, Takaesu G, Kobayashi T, Yoshimura A** 2007 Adaptor protein SH2-B linking receptor-tyrosine kinase and Akt promotes adipocyte differentiation by regulating peroxisome proliferator-activated receptor gamma messenger ribonucleic acid levels. Mol Endocrinol 21:1120-1131

57. **Obermeier A, Lammers R, Wiesmuller KH, Jung G, Schlessinger J, Ullrich A** 1993 Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. J Biol Chem 268:22963-22966

58. **Loeb DM, Stephens RM, Copeland T, Kaplan DR, Greene LA** 1994 A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase C-γ 1 abolishes NGF-promoted peripherin induction but not neurite outgrowth. J Biol Chem 269:8901-8910

59. **Obermeier A, Bradshaw RA, Seedorf K, Choidas A, Schlessinger J, Ullrich A** 1994 Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. EMBO J 13:1585-1590

60. **Vetter ML, Martin-Zanca D, Parada LF, Bishop JM, Kaplan DR** 1991 Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc Nat Acad Sci USA 88:5650-5654

61. **Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR** 1994 Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ 1 to mediate NGF responses. Neuron 12:691-705

62. **Maures TJ, Chen L, Carter-Su C** 2009 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 Mol Endocrinol 23:1077-1091

63. **Qian X, Ginty DD** 2001 SH2-B and APS are multimeric adapters that augment TrkA signaling. Mol Cell Biol 21:1613-1620

64. **Suzuki K, Mizutani M, Hitomi Y, Kizaki T, Ohno H, Ishida H, Haga S, Koizumi S** 2002 Association of SH2-B to phosphorylated tyrosine residues in the activation loop of TrkB. Res Commun Mol Pathol Pharmacol 111:27-39

65. **Maures TJ, Su H-W, Argetsinger LA, Grinstein S, Carter-Su C** 2011 Phosphorylation controls a dual function polybasic NLS in the adapter protein SH2B1β to regulate its cellular function and distribution between the plasma membrane, cytoplasm and nucleus. J Cell Sci 124:1542-1552

66. **Chen L, Carter-Su C** 2004 Adapter protein SH2-Bβ undergoes nucleocytoplasmic shuttling: implications for nerve growth factor induction of neuronal differentiation. Mol Cell Biol 24:3633-3647

67. **Chen L, Maures TJ, Jin H, Huo JS, Rabbani SA, Schwartz J, Carter-Su C** 2008 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. Mol Endocrinol 22:454-476

68. **Nordstrom LA, Lochner J, Yeung W, Ciment G** 1995 The metalloproteinase stromelysin-1 (transin) mediates PC12 cell growth cone invasiveness through basal laminae. Mol Cell Neurosci 6:56-68

69. **Farias-Eisner R, Vician L, Silver A, Reddy S, Rabbani SA, Herschman HR** 2000 The urokinase plasminogen activator receptor (UPAR) is preferentially induced by nerve growth factor in PC12 pheochromocytoma cells and is required for NGF-driven differentiation. J Neurosci 20:230-239

70. **Sternlicht MD, Werb Z** 2001 How matrix metalloproteinases regulate cell behavior. Ann Rev Cell Dev Biol 17:463-516

71. **Farias-Eisner R, Vician L, Reddy S, Basconcillo R, Rabbani SA, Wu YY, Bradshaw RA, Herschman HR** 2001 Expression of the urokinase plasminogen activator receptor is transiently required during "priming" of PC12 cells in nerve growth factor-directed cellular differentiation. J Neurosci Res 63:341-346

72. **Basbaum CB, Werb Z** 1996 Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. Curr Opin Cell Biol 8:731-738

73. **Ossowski L, Aguirre-Ghiso JA** 2000 Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. Curr Opin Cell Biol 12:613-620

74. **Vician L, Basconcillo R, Herschman HR** 1997 Identification of genes preferentially induced by nerve growth factor versus epidermal growth factor in PC12 pheochromocytoma cells by means of representational difference analysis. J Neurosci Res 50:32-43

75. **Koshelnick Y, Ehart M, Hufnagl P, Heinrich PC, Binder BR** 1997 Urokinase receptor is associated with the components of the JAK1/STAT1 signaling pathway and leads to activation of this pathway upon receptor clustering in the human kidney epithelial tumor cell line TCL-598. J Biol Chem 272:28563-28567

76. **Nguyen DH, Hussaini IM, Gonias SL** 1998 Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular signal-regulated kinase 1 and 2 which is required for increased cellular motility. J Biol Chem 273:8502-8507

77. **Resnati M, Guttinger M, Valcamonica S, Sidenius N, Blasi F, Fazioli F** 1996 Proteolytic cleavage of the urokinase receptor substitutes for the agonist-induced chemotactic effect. EMBO J 15:1572-1582

78. **Busso N, Masur SK, Lazega D, Waxman S, Ossowski L** 1994 Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol 126:259-270

79. **Del Rosso M, Anichini E, Pedersen N, Blasi F, Fibbi G, Pucci M, Ruggiero M** 1993 Urokinase-urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. Biochem Biophys Res Commun 190:347-352

80. **Anichini E, Zamperini A, Chevanne M, Caldini R, Pucci M, Fibbi G, Del Rosso M** 1997 Interaction of urokinase-type plasminogen activator with its receptor rapidly induces activation of glucose transporters. Biochemistry 36:3076-3083

81. **Goretzki L, Mueller BM** 1997 Receptor-mediated endocytosis of urokinase-type plasminogen activator is regulated by cAMP-dependent protein kinase. J Cell Sci 110 (Pt 12):1395-1402

82. **Cao D, Mizukami IF, Garni-Wagner BA, Kindzelskii AL, Todd III RF, Boxer LA, Petty HR** 1995 Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. Possible roles of complement receptor type 3 and calcium. J Immunol 154:1817-1829

83. **Vilhardt F, Nielsen M, Sandvig K, van Deurs B** 1999 Urokinase-type plasminogen activator receptor is internalized by different mechanisms in polarized and nonpolarized Madin-Darby canine kidney epithelial cells. Mol Biol Cell 10:179-195

84. **Wei Y, Yang X, Liu Q, Wilkins JA, Chapman HA** 1999 A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. J Cell Biol 144:1285-1294

85. **Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA** 1996 Regulation of integrin function by the urokinase receptor. Science 273:1551-1555

86. **Fujita K, Lazarovici P, Guroff G** 1989 Regulation of the differentiation of PC12 pheochromocytoma cells. Environ Health Perspect 80:127-142

87. **Greene LA, Tischler AS** 1976 Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424-2428

88. **Zhou T, Xu B, Que H, Lin Q, Lv S, Liu S** 2006 Neurons derived from PC12 cells have the potential to develop synapses with primary neurons from rat cortex. Acta Neurobiol Exp (Wars) 66:105-112

89. **Huang EJ, Reichardt LF** 2003 Trk receptors: roles in neuronal signal transduction. Ann Rev Biochem 72:609-642

90. **Meakin SO** 2000 Nerve growth factor receptors and mechanisms of intracellular signal transduction. Recent Res Dev Neurochem 3:75-91

91. **Wells A** 1999 EGF receptor. Int J Biochem Cell Biol 31:637-643

92. **Huff K, End D, Guroff G** 1981 Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. J Cell Biol 88:189-198

93. **Heasley LE, Johnson GL** 1992 The β-PDGF receptor induces neuronal differentiation of PC12 cells. Mol Biol Cell 3:545-553

94. **Traverse S, Gomez N, Paterson H, Marshall C, Cohen P** 1992 Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. Biochem J 288:351-355

95. **Nguyen TT, Scimeca JC, Filloux C, Peraldi P, Carpentier JL, Van Obberghen E** 1993 Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor. J Biol Chem 268:9803-9810

96. **Marshall CJ** 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185

97. **Cowley S, Paterson H, Kemp P, Marshall CJ** 1994 Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77:841-852

98. **Pang L, Sawada T, Decker SJ, Saltiel AR** 1995 Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270:13585-13588

99. **Kao S, Jaiswal RK, Kolch W, Landreth GE** 2001 Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. J Biol Chem 276:18169-18177

100. **York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJS** 1998 Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622-626

101. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-432

102. **Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, Hughes IA, McCamish MA, O'Rahilly S** 1999 Effects of recombinant leptin therapy in a child with congenital leptin deficiency. N Eng J Med 341:879-884

103. **Myers Jr. MG, Leibel RL, Seeley RJ, Schwartz MW** 2010 Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol Metab 21:643-651

104. **Chua Jr. S** 2010 SH2B1--the adaptor protein that could. Endocrinology 151:4100-4102

105. **Robertson SA, Leinninger GM, Myers Jr. MG** 2008 Molecular and neural mediators of leptin action. Physiol Behav 94:637-642

106. **Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S** 2003 Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. N Eng J Med 348:1085-1095

107. **Bochukova EG, Huang N, Keogh J, Henning E, Purmann C, Blaszczyk K, Saeed S, Hamilton-Shield J, Clayton-Smith J, O'Rahilly S, Hurles ME, Farooqi IS** 2010 Large, rare chromosomal deletions associated with severe early-onset obesity. Nature 463:666-670

108. **Rui L, Carter-Su C** 1999 Identification of SH2-Bβ as a potent cytoplasmic activator of the tyrosine kinase Janus kinase 2. Proc Natl Acad Sci USA 96:7172-7177

109. **Rui L, Herrington J, Carter-Su C** 1999 SH2-B, a membrane-associated adapter, is phosphorylated on multiple serines/threonines in response to nerve growth factor by kinases within the MEK/ERK cascade. J Biol Chem 274:26485-26492

110. **Duan C, Li M, Rui L** 2004 SH2-B promotes insulin receptor substrate 1 (IRS1)- and IRS2-mediated activation of the phosphatidylinositol 3-kinase pathway in response to leptin. J Biol Chem 279:43684-43691

111. **Cole TJ, Freeman JV, Preece MA** 1995 Body mass index reference curves for the UK, 1990. Arch Dis Child 73:25-29

112. **Doche MD, Bochukova EG, Su HW, Pearce L, Keogh JM, Henning E, Cline JM, Dale A, Cheetham T, Barroso I, Argetsinger LS, O'Rahilly SO, Rui L, Carter-Su C, Farooqi IS** 2012 *SH2B1* mutations are associated with maladaptive behavior and obesity. J Clin Invest 122:4732-4736

113. **Rui L, Gunter DR, Herrington J, Carter-Su C** 2000 Differential binding to and regulation of JAK2 by the SH2 domain and N-terminal region of SH2-Bβ. Mol Cell Biol 20:3168-3177

114. **Takagi M, Absalon MJ, McLure KG, Kastan MB** 2005 Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell 123:49-63

115. **Robbins J, Dilworth SM, Laskey RA, Dingwall C** 1991 Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 64:615-623

116. **Suzuki H, Arakawa Y, Ito M, Saito S, Takeda N, Yamada H, Horiguchi-Yamada J** 2007 MLF1-interacting protein is mainly localized in nucleolus through N-terminal bipartite nuclear localization signal. Anticancer Res 27:1423-1430

117. **Stoykova AS, Dabeva MD, Dimova RN, Hadjiolov AA** 1985 Ribosome biogenesis and nucleolar ultrastructure in neuronal and oligodendroglial rat brain cells. J Neurochem 45:1667-1676

118. **Scheer U, Benavente R** 1990 Functional and dynamic aspects of the mammalian nucleolus. Bioessays 12:14-21

119. **Pederson T** 1998 The plurifunctional nucleolus. Nucleic Acids Res 26:3871-3876

120. **Visintin R, Amon A** 2000 The nucleolus: the magician's hat for cell cycle tricks. Curr Op Cell Biol 12:372-377

121. **Maggi Jr. LB, Weber JD** 2005 Nucleolar adaptation in human cancer. Cancer Invest 23:599-608

122. **Hiscox JA** 2002 The nucleolus--a gateway to viral infection? Arch Virol 147:1077-1089

123. **Hetman M, Pietrzak M** 2012 Emerging roles of the neuronal nucleolus. Trends Neurosci 35:305-314

124. **Schmidt-Zachmann MS, Nigg EA** 1993 Protein localization to the nucleolus: a search for targeting domains in nucleolin. J Cell Sci 105 (Pt 3):799-806

125. **Davidson MB** 1987 Effect of growth hormone on carbohydrate and lipid metabolism. Endocrinology Rev 8:115-131

126. **Nyberg G, Bostrom S, Johansson R, Smith U** 1980 Reduced glucose incorporation to triglycerides following chronic exposure of human fat cells to growth hormone. Acta Endocrinol 95:129-133

127. **del Rincon JP, Iida K, Gaylinn BD, McCurdy CE, Leitner JW, Barbour LA, Kopchick JJ, Friedman JE, Draznin B, Thorner MO** 2007 Growth hormone regulation of p85α expression and phosphoinositide 3-kinase activity in adipose tissue: mechanism for growth hormone-mediated insulin resistance. Diabetes 56:1638-1646

128. **Berryman DE, List EO, Coschigano KT, Behar K, Kim JK, Kopchick JJ** 2004 Comparing adiposity profiles in three mouse models with altered GH signaling. GH IGF Res 14:309-318

129. **Ezzat S, Forster MJ, Berchtold P, Redelmeier DA, Boerlin V, Harris AG** 1994 Acromegaly. Clinical and biochemical features in 500 patients. Medicine (Baltimore) 73:233-240

130. **Goodman HM, Tai LR, Chipkin SR** 1990 The isoquinoline sulfonamide inhibitors of protein phosphorylation, H-7, H-8, and HA-1004, also inhibit RNA synthesis: studies on responses of adipose tissue to growth hormone. Endocrinology 126:441-450

131. **Yang S, Mulder H, Holm C, Eden S** 2004 Effects of growth hormone on the function of beta-adrenoceptor subtypes in rat adipocytes. Obesity Res 12:330-339

132. **Ridderstrale M, Amstrup J, Hilton DJ, Billestrup N, Tornqvist H** 2003 SOCS-3 is involved in the downregulation of the acute insulin-like effects of growth hormone in rat adipocytes by inhibition of Jak2/IRS-1 signaling. Horm Metab Res 35:169-177

133. **Tai P-KK, Liao J-F, Chen EH, Dietz JJ, Schwartz J, Carter-Su C** 1990 Differential regulation of two glucose transporters by chronic growth hormone treatment of cultured 3T3-F442A adipose cells. J Biol Chem 265:21828-21834

134. **Brown RJ, Adams JJ, Pelekanos RA, Wan Y, McKinstry WJ, Palethorpe K, Seeber RM, Monks TA, Eidne KA, Parker MW, Waters MJ** 2005 Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. Nat Struct Mol Biol 12:814-821

135. **Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C** 1993 Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237-244

136. **Argetsinger LS, Kouadio J-L, Steen H, Stensballe A, Jensen ON, Carter-Su C** 2004 Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. Mol Cell Biol 24:4955-4967

137. **Hansen LH, Wang X, Kopchick JJ, Bouchelouche P, Nielsen JH, Galsgaard ED, Billestrup N** 1996 Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and Stat5 activation. J Biol Chem 271:12669-12673

138. Smit LS, Meyer DJ, Argetsinger LS, Schwartz J, Carter-Su C 1999 Molecular events in growth hormone-receptor interaction and signaling. In: Kostyo JL ed. Handbook of Physiology. New York: Oxford University Press; 445-480

139. **Smit LS, Meyer DJ, Billestrup N, Norstedt G, Schwartz J, Carter-Su C** 1996 The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. Mol Endocrinology 10:519-533

140. **Wang X, Darus CJ, Xu BC, Kopchick JJ** 1996 Identification of growth hormone receptor (GHR) tyrosine residues required for GHR phosphorylation and JAK2 and STAT5 activation. Mol Endocrinology 10:1249-1260

141. **Smit LS, VanderKuur JA, Stimage A, Han Y, Luo G, Yu-lee L, Schwartz J, Carter-Su C** 1997 Growth hormone-induced tyrosyl phosphorylation and DNA binding activity of Stat5A and Stat5B. Endocrinology 138:3426-3434

142. **Zhu T, Goh EL, Graichen R, Ling L, Lobie PE** 2001 Signal transduction via the growth hormone receptor. Cell Signal 13:599-616

143. **Takaki S, Watts JD, Forbush KA, Nguyen NT, Hayashi J, Alberola-Ila J, Aebersold R, Perlmutter RM** 1997 Characterization of Lnk. An adaptor protein expressed in lymphocytes. J Biol Chem 272:14562-14570

144. **Velazquez L, Cheng AM, Fleming HE, Furlonger C, Vesely S, Bernstein A, Paige CJ, Pawson T** 2002 Cytokine signaling and hematopoietic homeostasis are disrupted in *Lnk*-deficient mice. J Exp Med 195:1599-1611

145. Dietz J, Schwartz J 1991 Growth hormone alters lipolysis and lipase activity in 3T3-F442A adipocytes. Metabolism, 1991

146. **Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A** 1992 The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401-410

147. **Erickson RL, Hemati N, Ross SE, MacDougald OA** 2001 p300 coactivates the adipogenic transcription factor CCAAT/enhancer-binding protein α. J Biol Chem 276:16348-16355

148. **Miller AD, Rosman GJ** 1989 Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980-982, 984-986, 989-990

149. **Lee DK, George SR, O'Dowd BF** 2006 Unravelling the roles of the apelin system: prospective therapeutic applications in heart failure and obesity. Trends Pharmacol Sci 27:190-194

150. **Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, Hinuma S, Baba A** 2004 Apelin is a novel angiogenic factor in retinal endothelial cells. Biochem Biophys Res Comm 325:395-400

151. **Beltowski J** 2006 Apelin and visfatin: unique "beneficial" adipokines upregulated in obesity? Med Sci Monit 12:RA112-RA119

152. **Rayalam S, Della-Fera MA, Krieg PA, Cox CM, Robins A, Baile CA** 2008 A putative role for apelin in the etiology of obesity. Biochem Biophys Res Commun 368:815-819

153. **Han S, Wang G, Qi X, Englander EW, Greeley Jr. GH** 2008 Involvement of a STAT3 binding site in inflammation-induced enteric apelin expression. Am J Physiol - Liver Physiol

154. **Kralisch S, Lossner U, Bluher M, Paschke R, Stumvoll M, Fasshauer M** 2007 Growth hormone induces apelin mRNA expression and secretion in mouse 3T3-L1 adipocytes. Regul Pept 139:84-89

155. **Huang TH, Oka T, Asai T, Okada T, Merrills BW, Gertson PN, Whitson RH, Itakura K** 1996 Repression by a differentiation-specific factor of the human cytomegalovirus enhancer. Nucleic Acids Res 24:1695-1701

156. **Amano K, Hata K, Muramatsu S, Wakabayashi M, Takigawa Y, Ono K, Nakanishi M, Takashima R, Kogo M, Matsuda A, Nishimura R, Yoneda T** 2011 Arid5a cooperates with Sox9 to stimulate chondrocyte-specific transcription. Mol Biol Cell 22:1300-1311

157. **Wilsker D, Patsialou A, Dallas PB, Moran E** 2002 ARID proteins: a diverse family of DNA binding proteins implicated in the control of cell growth, differentiation, and development. Cell Growth Differ 13:95-106

158. **Lahoud MH, Ristevski S, Venter DJ, Jermiin LS, Bertoncello I, Zavarsek S, Hasthorpe S, Drago J, de Kretser D, Hertzog PJ, Kola I** 2001 Gene targeting of Desrt, a novel ARID class DNA-binding protein, causes growth retardation and abnormal development of reproductive organs. Genome Res 11:1327-1334

159. **Whitson RH, Tsark W, Huang TH, Itakura K** 2003 Neonatal mortality and leanness in mice lacking the ARID transcription factor Mrf-2. Biochem Biophys Res Comm 312:997-1004

160. **Georgescu SP, Li JH, Lu Q, Karas RH, Brown M, Mendelsohn ME** 2005 Modulator recognition factor 1, an AT-rich interaction domain family member, is a novel corepressor for estrogen receptor α. Mol Endocrinol 19:2491-2501

161. **Tollet-Egnell P, Flores-Morales A, Stavreus-Evers A, Sahlin L, Norstedt G** 1999 Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. Endocrinology 140:3693-3704

162. **Yoshimura A, Ohkubo T, Kiguchi T, Jenkins NA, Gilbert DJ, Copeland NG, Hara T, Miyajima A** 1995 A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. EMBO J 14:2816-2826

163. **Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ** 1997 A family of cytokine-inducible inhibitors of signalling. Nature 387:917-921

164. **Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, Akira S, Kishimoto T** 1997 Structure and function of a new STAT-induced STAT inhibitor. Nature 387:924-929

165. **Matsumoto A, Masuhara M, Mitsui K, Yokouchi M, Ohtsubo M, Misawa H, Miyajima A, Yoshimura A** 1997 CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood 89:3148-3154

166. **Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, Tessarollo L** 1999 Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. Proc Natl Acad Sci USA 96:15239-15244

167. **Kernie SG, Liebl DJ, Parada LF** 2000 BDNF regulates eating behavior and locomotor activity in mice. EMBO J 19:1290-1300

168. **Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R** 2001 Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Mol Endocrinol 15:1748-1757

169. **Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, Reichardt LF** 2003 Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nature Neurosci 6:736-742

170. **Segal RA, Greenberg ME** 1996 Intracellular signaling pathways activated by neurotrophic factors. Ann Rev Neurosci 19:463-489

171. **Zhao J, Yuan X, Frodin M, Grummt I** 2003 ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. Molec cell 11:405-413