MOLECULAR MECHANISMS OF GROWTH HORMONE-INDUCED SIGNAL TRANSDUCTION AND SH2B1β-MEDIATED REGULATION OF THE ACTIN CYTOSKELETON

by

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To my father, Gary Lanning, the best biology teacher I ever had.

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Abstract

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Chair: Christin Carter-Su

Growth hormone (GH) regulates overall body growth and metabolism and is used therapeutically for a variety of clinical applications. GH binding to its receptor activates the tyrosine kinase, JAK2. Active JAK2 initiates multiple cellular responses to GH, including regulation of the cytoskeleton, that lead to cellular proliferation, differentiation and migration. Recent studies questioned whether JAK2 is the primary kinase responsible for transducing all GH signals. This thesis establishes that JAK2 is the primary kinase responsible for GH-mediated activation of Stat1, Stat3, Stat5, ERK1/2 and Akt in two established models of GH signaling, 3T3-F442A preadipocytes and H4IIE hepatoma cells. This work also characterizes two novel mechanisms by which the adaptor protein, SH2B1β, may modulate GH-induced regulation of the actin

cytoskeleton. The cytoskeleton scaffolding protein, βII-spectrin, was identified as an SH2B1β interacting protein. JAK2 was found to phosphorylate βII-spectrin in an SH2B1β-dependent manner. GH induced formation of a βII-spectrin/SH2B1β/JAK2 complex in 3T3-F442A cells and induced a shift in βII-spectrin subcellular localization in H4IIE cells. These results suggest that GH may regulate the cytoskeleton through an SH2B1β/βII-spectrin interaction. SH2B1β was also found to localize to focal adhesions, which are cytoskeletal structures that regulate cell anchorage and motility. GH increased the dynamic cycling of SH2B1β into and out of focal adhesions. In addition, PMA induced redistribution of SH2B1β out of focal adhesions. Two serines within SH2B1β (serines 161 and 165) were implicated in regulating this PMA effect as well as the dynamic cycling of SH2B1β into and out of focal adhesions. Mutation of SH2B1β serine 165 to glutamate also increased the overall focal adhesion number in cells. These results implicate SH2B1β as a novel focal adhesion protein and suggest that stimuli that induce phosphorylation of SH2B1β at serines 161 and/or 165 regulate SH2B1β dynamics at focal adhesions and may contribute to the regulation of overall focal adhesion number. Taken together, the work in this thesis demonstrates that GH activates downstream signaling through JAK2 in 3T3-F442A and H4IIE cells. This work also provides evidence that GH regulates the cytoskeleton through an SH2B1β/βII-spectrin interaction and/or modulating SH2B1β at focal adhesions.

Chapter 1

Introduction

Growth hormone (GH) is a major regulatory factor of overall body growth as evidenced by the height extremes in people with abnormal circulating GH levels or GH receptor (GHR) disruptions. GH also affects metabolism, cardiac and immune function, mental agility and aging. Currently, GH is being used therapeutically for a variety of clinical conditions including promotion of growth in short statured children, treatment of adults with GH deficiency and HIV-associated wasting. At the cellular level, GH either directly or indirectly elicits a variety of responses depending on cell type and context. These responses include cellular differentiation of epiphyseal prechondrocytes into chondrocytes and 3T3-F442A preadipocytes into mature adipocytes, chemotaxis of monocytes, and migration and proliferation of models of breast and endometrial cancer. To help reveal previous unrecognized functions of GH, better understand the known functions of GH, and avoid adverse consequences that are often associated with exogenous GH administration, careful delineation of the molecular mechanisms whereby GH induces its diverse effects is needed.

Growth Hormone Signal Transduction

GH is a peptide hormone that is secreted into the circulation by the anterior pituitary and acts upon various target tissues expressing GHR. GH binding to GHR activates the tyrosine kinase Janus kinase 2 (JAK2), thus initiating a multitude of

signaling cascades that result in a variety of biological responses including cellular proliferation, differentiation and migration, prevention of apoptosis, cytoskeletal reorganization and regulation of metabolic pathways. A number of signaling proteins and pathways activated by GH have been identified, including JAKs, signal transducers and activators of transcription (Stats), the mitogen activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3'-kinase (PI3K) pathway. Although these signal transduction pathways have been well characterized, the manner by which GH activates these pathways, the downstream signals induced by these pathways, and the cross-talk with other pathways are not completely understood.

GH receptor dimerization and activation

The downstream signaling pathways mediated by the GHR are initiated upon the binding of GH to the extracellular domain of the GHR (Fig. 1.1A). Early analysis of the extracellular domain of GHR in association with GH indicated that one GH molecule binds sequentially to two GHR molecules (1). Formation of this GH-GHR_2 trimer complex was thought to be necessary and sufficient for GH responses (1). However, other studies indicated that dimerization of the GHR was insufficient for activation of GH-mediated signaling (2) and that preformed GHR dimers exist prior to GH binding (3). These findings, along with newer results from tests of GHR activation, lay the groundwork for a revised model of GHR activation. A recent study (4) confirmed that unliganded GHR exists as a dimer, using co-immunoprecipitation, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and X-ray crystallography of the extracellular domain of GHR. Only minor differences were observed between the crystal structures of the liganded and unliganded GHR extracellular domain dimers. However, inducing a nominal 40° clockwise rotation in the lower α-helical transmembrane sequence by insertion of alanine residues or a nominal

100° clockwise rotation in the juxtamembrane helix of GHR just N-terminal of the Box 1 domain containing the JAK2 binding site resulted in constitutive activation of JAK2 and Stat5. From these and other findings, Brown et al. (4) suggested a model whereby GH binding asymmetrically at the receptor binding sites of preformed GHR dimers causes the intracellular domains of the GHR to undergo relative rotation. Because the cytoplasmic domain of each GHR molecule is thought to bind a single JAK2 molecule, this rotation is postulated to bring two JAK2 molecules into sufficient proximity to allow each JAK2 molecule to phosphorylate the activating tyrosine residue in the kinase domain on the other JAK2 molecule, thereby activating JAK2. Since GH binding has also been reported to increase the co-immunoprecipitation of JAK2 with GHR (5), the GH-induced conformational change in GHR may also increase the stability of the GHR-JAK2 interaction. More recently, Yang et. al (6) provided evidence using GHR truncation and point mutants and GHR-low density lipoprotein receptor (LDLR) chimera receptors suggesting that the dimerization interface is the main contributor to GHR predimerization rather than the length of the receptor transmembrane domain. However, they also found that GHR-LDLR receptor chimeras were less sensitive to inhibition of GH signaling by a GHR antibody and less sensitive to induced receptor metalloprotease cleavage. Therefore, they concluded that the longer transmembrane domain of the GHR compared to the GHR-LDLR receptor chimera does provide added sensitivity to GH-induced JAK2 activation and presumably, downstream signaling. Finally, in a subsequent study, this group presented data in opposition to the prevailing theory that one GH molecule containing two asymmetric binding sites binds one GHR dimer (6). Here, Yang et. al showed GHR and JAK2 activation in response to recombinantly produced GH-GH and G120R-G120R dimers. G120R is a naturally occurring GH mutant lacking one GHR binding site, rendering the molecule unable to activate GHR-mediated signaling (Reviewed in (7)). This study showed that even though G120R-

G120R molecules contain only one of the two known GHR asymmetric binding sites, the dimer molecule was still able to activate JAK2 and Stat5. While the physiological significance of this study remains in question, these results, along with the studies describing new models of GHR dimerization and activation, are suitable reminders that ongoing research is needed to more precisely define the mechanism(s) initiating GH signaling.

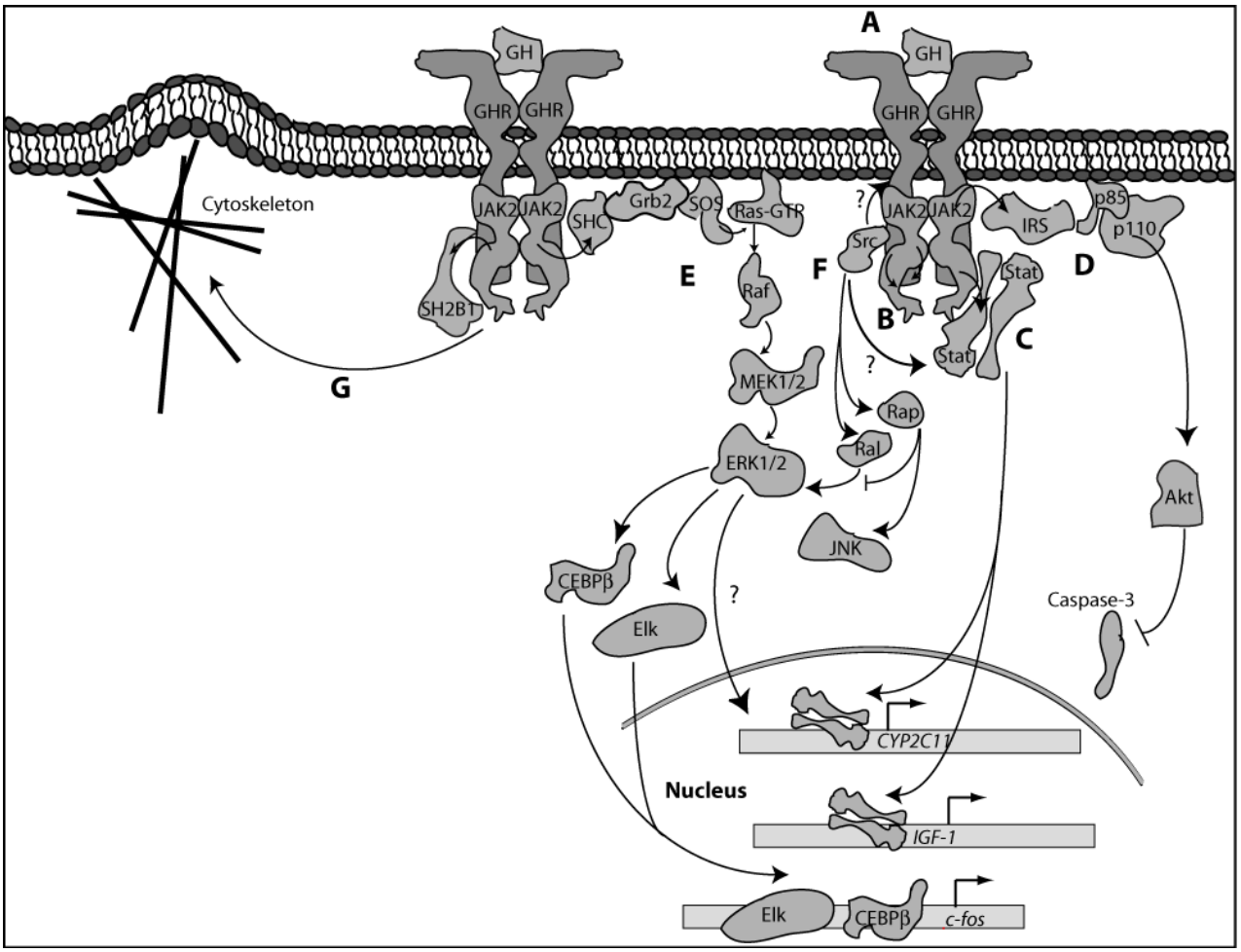

Figure 1.1 Signal transduction pathways induced by growth hormone.
(A) GH binds a GHR dimer, inducing a conformational change that activates two JAK2 molecules. (B) JAK2 activation induces JAK2 autophosphorylation and JAK2 phosphorylation on multiple GHR tyrosines. GH-activated JAK2 also phosphorylates and activates multiple signaling proteins and pathways including (C) Stats, (D) IRS and PI-3 kinase and (E) MAPK. (F) The binding of GH to GHR may also activate Src tyrosine kinase, initiating other signaling pathways. (G) GH induces changes in cytoskeletal dynamics.

GH signal transduction via JAK2

Activation of JAK2 is thought to be the key step in initiating GH signaling (5). The FERM domain of JAK2 is thought to mediate JAK2 binding to the cytoplasmic Box 1 region of GHR (8-10). Following GH activation of JAK2, JAK2 autophosphorylates multiple tyrosines (11, 12) and subsequently phosphorylates multiple tyrosine residues in GHR (13, 14) (Fig. 1A). Based upon JAK2 overexpression systems, some of the autophosphorylation sites in JAK2 appear to be regulatory sites since mutating them has been shown to either stimulate (e.g. tyrosine 119, 570) or decrease (e.g. tyrosines 221, 972, 1007) JAK2 activity (11, 15-18). For some of those tyrosines, phosphorylation is thought to cause a conformational change in JAK2 that alters JAK2 activity. For example, phosphorylation of tyrosine 1007 is thought to expose the substrate and/or ATP binding sites (15) whereas phosphorylation of tyrosine 119 is thought to promote dissociation of JAK2 from its associated cytokine family receptor (18). Autophosphorylation of some tyrosines are alternatively or additionally thought to regulate JAK2 activity indirectly by recruiting regulatory proteins to JAK2. For example, phosphorylated tyrosine 1007 has also been shown to bind the negative regulators of cytokine signaling SOCS1 (19), SOCS3 (20) and the phosphatase PTP1B (21) (discussed in more detail below). Autophosphorylation of tyrosine 813 appears to enhance JAK2 activity as a consequence of recruiting the adaptor protein, SH2B1 (also known as SH2-B or PSM1) (12). SH2B1 has been hypothesized to either stabilize the active conformation of JAK2 (22) or promote the dimerization of JAK2 (23). Some of the autophosphorylation sites in JAK2 (e.g. tyrosine 966 (24)) as well as the phosphorylated tyrosines in GHR, are thought to serve as docking sites for signaling molecules containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Based on mutational studies, 7 different tyrosines within the cytoplasmic domain of the GHR have been implicated in at least one downstream GH response (reviewed in (25)). For

example, 5 or 6 phosphorylated tyrosines in GHR have been hypothesized to bind Stat5a and Stat5b, based upon decreased GH-dependent Stat5 tyrosyl phosphorylation or Stat5-dependent responses in cells expressing mutated or truncated GHR (13, 14, 26-28). Recruitment of these signaling molecules to GHR-JAK2 complexes and their activation allows GH to elicit diverse biological and physiological effects. A number of signaling proteins and pathways are thought to be initiated at least in part as a consequence of binding to activated GHR-JAK2 complexes (reviewed in (25, 29)). Examples include pathways involving Stats 1, 3, 5a and 5b, the MAPK pathway, and the phosphatidylinositol 3'-kinase (PI3K) pathway (Fig. 1.1B and C).

GH signal transduction via Src tyrosine kinase

One of the more interesting recent developments in GH signaling is support for the hypothesis that not all GH signaling events lie downstream of JAK2. Zhu et al. (30) provide evidence using both pharmacological inhibitors and kinase inactive proteins in NIH3T3 cells that the tyrosine kinase Src is activated by GH independent of JAK2. Using the same reagents, the same group reported that full activation of the Ras-like small GTPases RalA, RalB, Rap1 and Rap2 by GH requires both c-Src and JAK2 (30, 31) (Fig. 1.1C) whereas activation of Stat5 requires only JAK2. Activation of RalA by GH was linked to increased phospholipase D activity and the formation of its metabolite, phosphatidic acid, which were in turn linked to GH-activation of extracellular regulated kinases (ERKs) 1 and 2 and subsequent Elk-1-mediated transcription (30), suggesting that GH activation of ERKs 1 and 2 is at least partially dependent upon GH activation of Src and independent of JAK2. GH-dependent Rap1 activity appears to be dependent on CrkII-C3G activation and capable of mediating CrkII enhancement of GH-stimulated JNK/SAPK activity. Rap1 was also implicated as an inhibitor of GH activation of RalA and its subsequent stimulation of ERKs 1 and 2 (31). The latter suggests that the

balance between Ral and Rap protein activation by GH would affect the relative levels of activation of ERKs 1/2 versus JNK/SAPK (Fig. 1.1C). Using a COS7 cell overexpression system, Manabe et al. (32) also found GH to modestly stimulate Src activity. They showed that Src can bind to and phosphorylate GHR and used Src inhibitor or anti-sense to implicate Src in GH-dependent tyrosyl phosphorylation of GHR and Stat5a/b but not JAK2 in F-36P human leukemia cells (Fig. 1.1C). Cell type specificity was one hypothesis put forward to explain the apparent discrepancy between these two groups regarding the role of Src in Stat5b phosphorylation (32). Previous studies in IM-9 and CHO cells based upon truncated and mutated GHR and JAK2 inhibitors had suggested that regulation of cellular [Ca^{2+}] by GH may also be JAK2 independent (reviewed in (25), raising the possibility that this function might also be mediated by Src. Although recent inhibitor studies by Zhang et al. (33) found that human GH-induced increases in cytosolic free Ca^{2+} in, and insulin secretion from, BRIN-BD11 beta cells appeared to be dependent upon activation of both JAK2 and Src, these actions were not mediated via the GHR but rather the prolactin receptor, which can also bind human GH. In contrast, the rise in cytosolic free Ca^{2+} elicited by bovine GH, which binds only to the GHR, was not blocked by inhibitors of either JAK2 or Src. Thus, these data support the GHR-mediated increase in cytosolic free Ca^{2+} being independent of JAK2. They also suggest that GH binding to the GHR activates an as yet unidentified, early signaling protein in addition to JAK2 and Src. Most recently, in an elegant study by Rowlinson et al. (34), a GH-induced conformational change in a loop (F'G' loop) of one of two β-sandwich modules in the extracellular domain of GHR was found to be responsible for activating the Src family kinase, Lyn, but not JAK2 in FDC-P1 cells. In combination with a second published report, this group also found that liver samples from mice harboring GHR mutations rendering the receptor unable to bind JAK2 still displayed Src and ERK1/2 activation in response to GH treatment (35). The conclusion from these studies was that

GH-induced activation of ERK1/2 is mediated through Src family kinase while STAT5 activation is mediated through JAK2, and that movement of the F'G' loop within the GHR dictates which signaling pathway will be activated. Collectively, these results indicate that both Src family kinases and JAK2 may transduce the GH signal. However it is not currently clear if both kinases are utilized in all cells, or whether the relative abundance of a particular kinase defines utilization. Therefore, a more complete understanding of the relative contribution of each kinase in different cell types remains an important question.

GH regulation of Stat transcription factors

A number of the responses to GH involve transcription factors and gene expression. Among these transcription factors, members of the SH2 domain-containing signal transducers and activators of transcription (Stat) family of proteins have been shown to be particularly important for JAK2-mediated GH signaling (36) and will be discussed below. The regulation of other transcription factors is outside the scope of this thesis, and is described elsewhere in detail (36, 37). Activation of Stat proteins is known to require tyrosine phosphorylation-dependent homo or heterodimerization, a process that is facilitated by the GH-dependent creation of Stat binding sites on the activated GHR-JAK2 complex (Fig. 1.1B). Once bound to these sites on the activated GHR-JAK2 complex, Stats 1, 3, 5a and 5b are thought to be phosphorylated by JAK2 after which they dimerize, translocate to the nucleus and act as transcription factors for many important GH-regulated genes (36, 38, 39). Many past and recent studies have shown that activation of Stat5a and Stat5b is critical for a variety of GH functions, including changes in metabolism, body growth and sex-dependent liver gene regulation (reviewed in (36, 39)).

Although Stat5a and Stat5b have been implicated in body growth via mouse gene deletion studies, only recently have Stat5b binding sites in the IGF-1 gene promoter elements been identified and shown to regulate IGF-1 transcription in a GH-dependent manner through Stat5b (40, 41). In support of Stat5 being important for GH-dependent IGF-I levels in serum and body growth, a patient with severe growth retardation and immunodeficiency has been found to have a mutation in the *Stat5b* gene that results in the loss of GH-induced tyrosyl phosphorylation of Stat5b (42) (Fig 1.2A).

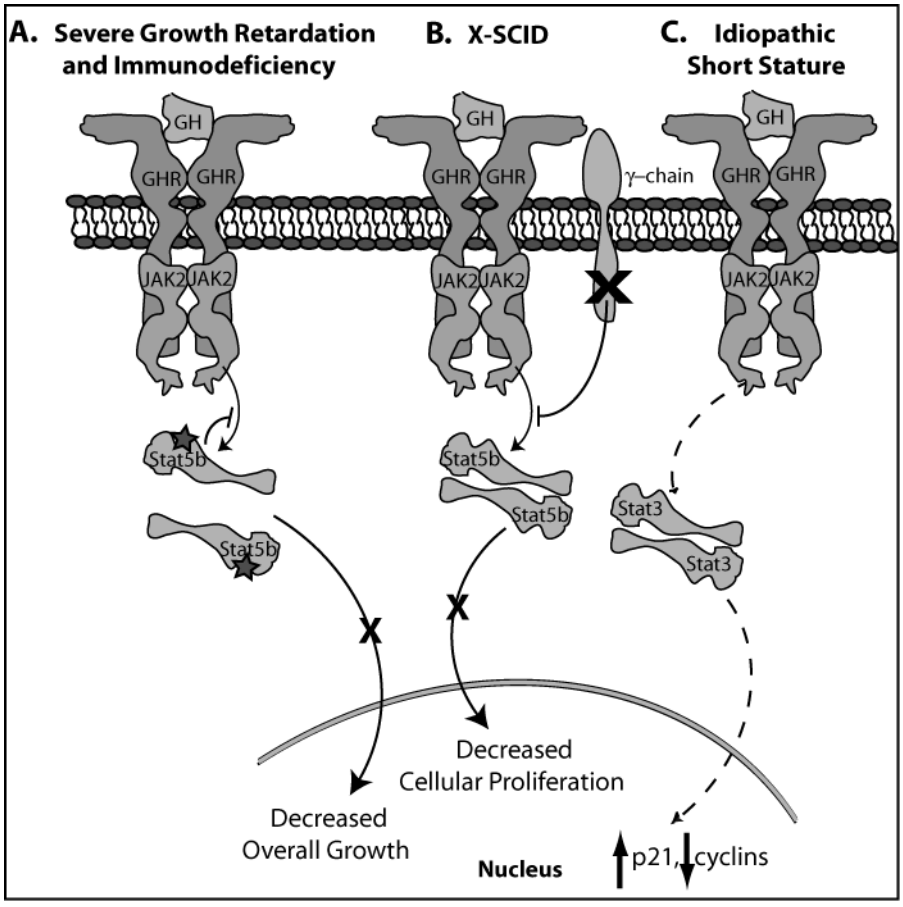

Figure 1.2. Diseases and disorders associated with abnormal GH-induced JAK-Stat signaling.

(A) A patient has recently been identified who has severe growth retardation and immunodeficiency as a result of a mutation in the Stat5b gene that abrogates JAK2-dependent phosphorylation and nuclear localization of Stat5b. (B) EVB-transformed lymphocytes from X-SCID patients lacking the common cytokine receptor (-chain exhibit decreased GH-induced JAK2-dependent phosphorylation and nuclear localization of Stat5b. (C) Fibroblasts from several patients exhibiting idiopathic short stature exhibit attenuated GH-induced JAK2-dependent phosphorylation and nuclear translocation of Stat3, increases in the cell cycle inhibtor, p21, and decreases in cyclins.

Recently, an exciting mechanism was proposed whereby Stat5 and the transcriptional repressor, B cell lymphoma 6 (Bcl6), interact to regulate GH-induced gene expression (43, 44). Using gene set enrichment analysis to identify novel GH-regulated genes at time points matching important physiological actions, Chen et. al

identified Bcl6 as being strongly inhibited by GH. They further found that upon GH stimulation, there was an increase in Stat5 occupancy on the highly GH-responsive gene, SOCS2 (see below for more on SOCS2). Concomitant with this, Chen et. al found a decrease in Bcl6 occupancy of the SOCS2 gene. Therefore, the results of this study for the first time identified GH-regulation of a powerful transcriptional repressor (Bcl6) and suggested that Stat5 and Bcl6 compete for regulation of the SOCS2 gene.

Although current dogma dictates that JAK2 phosphorylates Stat5 through direct interactions between GHR, JAK2 and Stat5, Adriani et al. (45) have recently demonstrated a requirement for the common cytokine receptor γ-chain (γ_c) for proper GH-mediated Stat5b activation in B cell lines. These investigators found that in EBV-transformed lymphocytes from γ_c negative X-SCID patients, GH was able to normally activate JAK2, but GH-dependent Stat5b phosphorylation and nuclear localization were significantly suppressed. These cells exhibited a total loss of GH-induced proliferation. Reconstitution of X-SCID patient lymphoblastoid cell lines with wild-type γ_c resulted in normal GH-induced phosphorylation of Stat5b and nuclear localization. This study suggests a novel dependence of GH signaling on the common cytokine receptor γ-chain in certain cell types, consistent with the presence of γ-chains in non-hematopoietic tissues and short stature of X-SCID patients (Fig. 1.2B). Whether this effect of γ-chain on GHR signaling is a direct or indirect effect remains to be determined.

Another clinical report in 2006 describes patients diagnosed with idiopathic short stature whose fibroblasts exhibit normal activation of Stats 5a and 5b but impaired activation of Stat3 (46) (Fig. 1.2C). Idiopathic short stature is characterized by a normal birth weight and no endocrine abnormalities but a retarded growth velocity and a height more than two standard deviations below the mean. In fibroblasts taken from these patients, GH-induced Stat3 activation was attenuated, cyclin levels were reduced and

levels of $p21^{WAF/CIPI}$ (a negative regulator of the cell cycle) were elevated (46). The idiopathic short stature phenotype and high $p21^{WAF/CIPI}$ levels in the human fibroblasts were reversed by treatment of patients with exogenous GH. Further studies are needed to know whether the elevated levels of $p21^{WAF/CIPI}$ and/or suppressed Stat3 activation are responsible for the short stature.

GH signal transduction via MAPK and PI3-kinase pathways

The Ras/MAPK pathway has also been shown to be activated by GH (reviewed in (25, 29)). GH has been shown to stimulate the binding of the adapter protein Shc to GHR-JAK2 complexes; the tyrosyl phosphorylation of Shc and its binding to Grb2 and the guanine nucleotide exchange factor, SOS; and the activity of Ras, Raf, mitogen-activated protein kinase/extracellular-regulated protein kinase (MEK) and finally ERKs 1 and 2 (47-50) (Fig. 1.1D). Although several groups have linked GH activation of ERKs 1 and 2 to JAK2, the Lobie laboratory have data suggesting that GH might also regulate ERKs 1 and 2 by a Src-dependent, JAK2-independent pathway that involves phospholipase D and RalA and RalB (30) or by a c-Src-FAK-Grb2 complex (reviewed in (29)). Alternatively, Yamauchi et al. (51) propose that GH activates the MAPK pathway by stimulating tyrosyl phosphorylation of a Grb2 binding site in the epidermal growth factor receptor. GH activation of the Ras/ERK pathway has been linked to GH activation of a variety of proteins (reviewed in (29)). Examples include phospholipase A2, which has been linked to GH-induced P450-catalyzed formation of an active arachidonic acid metabolite and expression of CYP2C12; and the transcription factor Elk1 whose phosphorylation by ERK1/2 is required for transcription via the *c-fos* serum response element (SRE). More recently, GH-induced ERK1/2 has been shown to phosphorylate the transcription factor CEBPβ, an event that has been implicated in CEBPβ nuclear translocation (52, 53) (Fig. 1.1C) and differentiation of 3T3-F442A preadipocytes into

adipocytes (54). Although Stat5b has been thought largely responsible for sex-dependent liver gene expression, including regulation of expression of the CYP2C11 gene (39), Verma et al. (55) raise the possibility that ERK1/2 may also regulate expression of CYP2C11 gene, based upon correlative data using different doses of pulsatile GH replacement therapy in mice (Fig 1.1C).

Recent results from Yang et al. (56) indicate that in 3T3-F442A cells, GHR is selectively enriched in caveolar and lipid raft domains of the plasma membrane. GH stimulation induced in this fraction accumulation and activation of Ras/MAPK, but not Stat signaling molecules. Disruption of these fractions using methyl-β-cycoldextrin inhibited GH-induced ERK1/2 activation, but had no effect on GH-stimulated Stat5 activation. These findings imply that GHR membrane localization may be important for the initiation of different GH-induced signal transduction pathways and that GH induction of ERKs 1/2 and Stat5 may require GHR in different cellular compartments.

In addition to activating the Ras/MAP kinase pathway, GH has also been shown to stimulate the PI-3 kinase pathway (reviewed in (29), Fig. 1.1B). One possible mechanism whereby GH activates PI-3 kinase is through tyrosyl phosphorylation of the large adaptor proteins designated insulin receptor substrate (IRS) proteins because of their known role in insulin signaling. GH stimulates the tyrosine phosphorylation of IRS-1, IRS-2 and IRS-3, and phosphorylation of these IRS proteins is known to lead to their association with multiple signaling molecules including the p85 subunit of PI-3 kinase. Other data suggest that GH might activate PI-3 kinase through a CrkII-IRS-1 interaction (57) or through binding of the p85α and p85β subunits of PI-3 kinase (58). Activation of PI3 kinase has been linked by inhibitor studies to GH stimulation of glucose transport (59) and the anti-apoptotic serine kinase Akt (60). This GH-dependent activation of Akt has been shown to be dependent on the presence of the JAK2 binding region of GHR,

and implicated in GH promotion of cell survival, possibly through inhibition of the pro-apoptotic caspase-3 protein (61). GH-induced activation of p70S6K, a kinase involved in the control of cell proliferation and differentiation, has also been shown to be activated in PI-3 kinase-dependent and PKC-dependent manners (62-64).

Negative regulators of GH signaling

GH is secreted episodically and GH responses are transient. In contrast, prolonged activation of JAK2 has been associated with cell transformation and cancer. Thus, precise regulation of GH signaling is vitally important for the proper maintenance of body growth and metabolism, with down-regulation of GH signaling being an important aspect of proper GH signaling. Current knowledge of down-regulation of the GH signal reviewed here includes blockage or removal of SH2 and PTB binding sites by inhibitory molecules or dephosphorylation, and ubiquitin-dependent GHR endocytosis.

Suppressor of cytokine signaling proteins

The suppressor of cytokine signaling (SOCS) family of proteins plays an important role in the negative regulation of GH signaling. There are eight members of the SOCS family, of which GH has been reported to induce the expression of four, namely, SOCS-1, -2, -3 and CIS (cytokine-inducible SH2-containing protein) (reviewed in (65)). SOCS proteins share a centrally located SH2 domain and a motif termed the SOCS box, which resides in the carboxy-terminus. Although it has been known for some time that the SOCS family is involved in negatively regulating GH signaling, more recent studies continue to shed light on the mechanisms of this regulation (Fig. 1.3). SOCS-1 is thought to bind the activating tyrosine in the kinase domain of JAK2 and inhibit JAK2 activity (19) (Fig. 1.3A). SOCS-3 has been shown to bind this residue in JAK2 as well as to phosphorylated residues in GHR (20, 66, 67). SOCS-3's mechanism

of GH signaling inhibition is thought to be by inhibition of JAK2 kinase activity through a mechanism dependent on SOCS-3 binding to GHR (Fig. 1.3A). SOCS-2 has been shown to bind phosphorylated GHR GST fusion proteins and peptides (66-68). CIS has also been shown to bind phosphorylated GST-GHR fusion proteins (66, 67). The SOCS-2 and CIS binding sites in GHR were subsequently mapped to tyrosines 487 and 595 using a mammalian 2-hybrid system (14). The mechanism of SOCS-2 and CIS inhibition of GH signaling may be through inhibiting Stat5b binding of GHR, however, in SOCS overexpression studies, SOCS-2 and CIS inhibition of GH signaling seems to be less effective than that of SOCS-1 and SOCS-3 (66) (Fig 1.3A). Additionally, while SOCS-2 and CIS bound GHR tyrosines 487 and 595 in the mammalian 2-hybrid system, Stat5a and Stat5b were found to bind GHR tyrosines 534, 566 and 627 (14). These results suggest that Stat5 does not compete with SOCS-2 or CIS for binding sites on GHR. SOCS-1, and possibly SOCS-2 and SOCS-3, also appear to be involved in the ubiquitination of the GHR-JAK2 complex as each have been shown to be associated with ubiquitin ligase activity (reviewed in (65), Fig. 1.3B). Results using pharmacological inhibitors of proteasomes and dominant negative forms of CIS indicate that CIS negatively regulates GHR signaling at least in part by stimulating GHR internalization and proteasomal degradation (69) (Fig 1.3B). New evidence indicates that SOCS-2 may also inhibit GH responses in the animal indirectly by antagonizing IGF-1 signaling (70). Mice lacking SOCS-2 are large (71), suggesting physiological relevance of SOCS-2 as a negative regulator of GHR. Although at high concentrations, SOCS-3 is able to down-regulate GH-induced JAK2 activity and has been shown to be associated with ubiquitin ligase activity, the physiological significance of SOCS-3 action on GH signaling is currently unclear because liver-specific SOCS-$3^{-/-}$ mice do not differ in size from wild-type littermates (72). Similarly, $CIS^{-/-}$ and SOCS-$1^{-/-}$ mice are not bigger than normal (73, 74). It is always possible that these SOCS proteins share some redundancy in function,

and that increased body size would be observed if they were deleted in combination. Because SOCS proteins are synthesized in response to other ligands, a number of recent studies have investigated whether GH insensitivity is a consequence of elevated levels SOCS protein. In that regard, Leung et al. (75) have implicated SOCS-2 upregulation in the known ability of estrogen to inhibit GH signaling. Similarly, increases in SOCS 1 and 3 have been implicated in the ability of sepsis to inhibit GH signaling in liver (76, 77), and increases in SOCS 2 and 3 in the negative effect of uremia on hepatic GH signaling and growth (78).

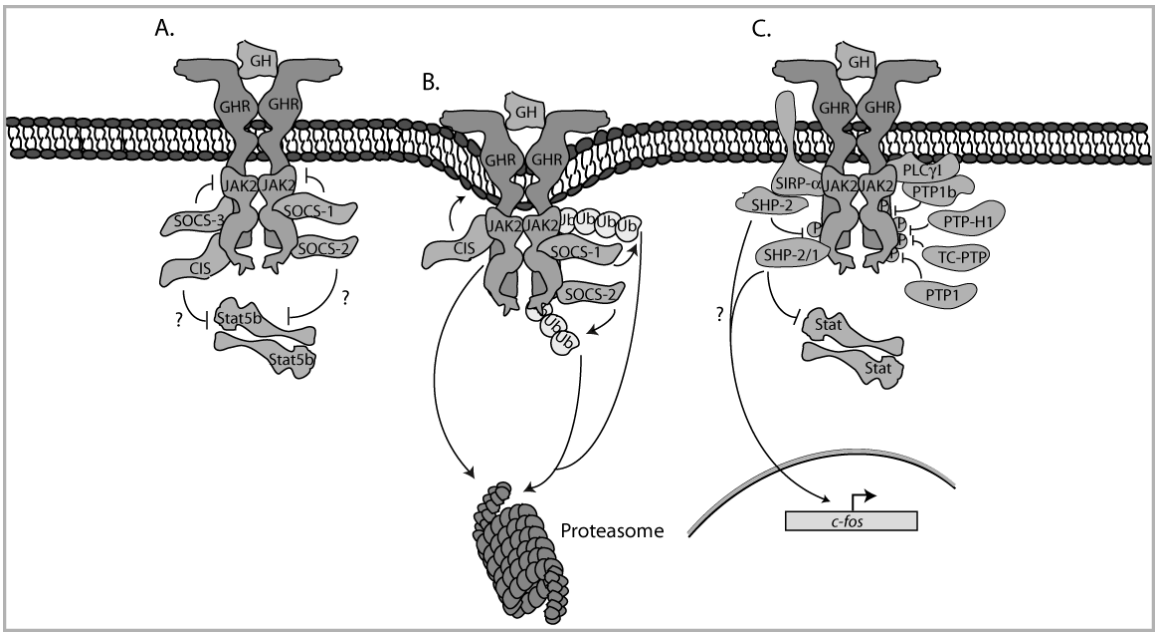

Figure 3. Negative regulation of GH signaling.
(A) SOCS-1 binds JAK2 and inhibits JAK2 kinase activity. SOCS-3 is thought to bind phosphorylated GHR and inhibit JAK2 kinase activity. SOCS-2 and CIS bind phosphorylated GHR and may compete with Stat5B for GHR binding sites. (B) Internalization and degredation of activated GHR may be facilitated by CIS and the ubiquitin ligase activity associated with SOCS proteins. (C) Protein tyrosine phosphatases negative regulate GH signaling by binding the activated receptor complex and presumably dephosphorylating phosphotyrosines on JAK2, GHR or associates signaling proteins. SHP2 has been hypothesized to be both a positive and negative regulator of GH signaling.

Protein tyrosine phosphatases

Another important mechanism whereby GH signaling is thought to be negatively regulated is through protein tyrosine phosphatases (PTPs). A number of phosphatases have been reported to down-regulate GH signaling, including SH2 domain-containing protein-tyrosine phosphatase (SHP-1), SHP-2, protein-tyrosine phosphatase (PTP)-H1, PTP1, TC-PTP and PTP1b (reviewed in (65), Fig. 1.3C). SHP-1 has been implicated as a negative regulator of GH signaling based upon the observation that GH-dependent tyrosyl phosphorylation of JAK2 and DNA binding of Stat5b are prolonged in liver extracts from motheaten mice deficient in SHP-1 (79). SHP-2 is reported to both positively and negatively regulate GH signaling. Based upon phosphatase inactive forms of SHP-2, Frank and colleagues (80) observed that overexpression of a catalytically inactive form of SHP-2 inhibited GH stimulation of *c-fos* enhancer-driven luciferase reporter, leading them to conclude that SHP-2 is a positive regulator of GH signaling. In contrast, Stofega et al. (81) reported that mutating the SHP-2 binding sites in GHR (Y595 and Y487) enhanced and prolonged GH-dependent tyrosyl phosphorylation of JAK2, GHR, and Stat5b, leading them to propose that SHP-2 is a negative inhibitor of GHR signaling. However, SOCS-2 and CIS are also reported to bind Y595 and Y487 (14). This raises the possibility that mutating these tyrosines in GHR prolongs GH-dependent tyrosyl phosphorylation of JAK2, GHR, and Stat5b due to the inability of SOCS-2 and CIS to inhibit signaling. Because the dominant negative forms of SHP-2 could affect multiple steps in GH signaling, not just JAK2 activity, and mutation of GHR binding sites for SHP-2 could also affect binding of other proteins to GHR, definitive resolution of the role of SHP-2 in GH signaling awaits further studies. Interestingly, Stofega et al (82) have also shown that GH stimulates the tyrosyl phosphorylation of the JAK2-associated membrane protein SIRP-α (signal regulatory

protein alpha). The phosphorylated tyrosines in SIRP-α recruit SHP-2 (Fig. 1.3C). Mutation of those tyrosines enhances GH signaling, suggesting that recruitment of SHP-2 to SIRPα in response to GH may also contribute to the attenuation of GH signaling (82). Pasquali et al. (83) have recently shown that PTP-H1, PTP1, TC-PTP, and PTP1b are all able to dephosphorylate GHR. PTP1b knockout mice also display increased JAK2, Stat5 and Stat3 phosphorylation in response to GH compared to wild type mice (84). Interestingly, Choi and colleagues (85) have recently demonstrated that phospholipase Cγ1 provides a physical link between JAK2 and PTP1b in a GH-dependent manner, leading to attenuation of GH-induced signaling. Why phospholipase Cγ1 would serve as an adapter protein for PTP1b is unclear.

Receptor internalization

The Strous laboratory has elucidated mechanisms whereby GHR is internalized in both ubiquitin-dependent and independent manners. They have identified a motif in the cytosolic domain of GHR that recruits the ubiquitin conjugation system to GHR (86). The recruitment of the ubiquitin conjugation system, as well as the activity of the proteasome, seem to be necessary for GHR internalization (87, 88). Although both an intact ubiquitin conjugation system and full proteasome activity seem to be required for subsequent proteasome-specific degradation of GHR, actual conjugation of ubiquitin to GHR does not seem to be necessary. The ubiquitination, internalization and degradation of the GHR/JAK2 complex have also recently been reported by Rico-Bautista et al. (89) to depend on an intact actin cytoskeleton.

Receptor processing and subcellular localization

Like amyloid precursor protein, Notch, and ErbB4, GHR appears to undergo "regulated intramembrane processing", or RIP (90). Following the same RIP program as the three receptors above, the extracellular domain of GHR is initially cleaved by the metalloprotease, tumor necrosis factor-(converting enzyme (TACE or ADAM-17), which results in shedding of the GHR extracellular domain (91). The remaining membrane-bound GHR is clipped within the lipid bi-layer, releasing the intracellular portion of GHR into the cytosol (92). The exact functional significance of the releasing of this domain of GHR into the cytosol is currently unknown. However, studies of other receptors undergoing RIP and those classically thought to be membrane-bound that are now being found in the cytosol may give hints as to the function of cytosolic GHR domain. Following RIP, ErbB4 has been shown to translocate to the nucleus where it plays a role in regulating transcription (93).

It is interesting to note that full-length GHR has been reported in the nucleus of various cell types. Using monoclonal antibodies specific for GHR, Lincoln et al. (94) identified GHR in the nucleus of a variety of normal and neoplastic cell types. Gevers et al. (95) found GHR present in the nucleus of both germinal and proliferating chondrocytes in the rat growth plate and Vespasiani Gentilucci et al. (88) found GHR in the nucleus of hepatocytes from patients in the later stages of chronic liver disease. Finally, Conway-Campbell found GHR to localize to the nucleus in response to GH stimulation in CHO-K1 cells and found nuclear GHR to be correlated with proliferation in a model of liver regenration (96). It will be interesting to see if the γ-secretase-processed intracellular domain of GHR also translocates to the nucleus and to determine the function of nuclear GHR.

Along with being targeted to the nucleus, GH and GHR have been reported in the mitochondria (97, 98). Perret-Vivancos et al. (99) recently reported that GH and GHR internalization through the caveolar pathway was essential for their targeting to the

mitochondria, and hypothesized that mitochondrial targeting was required for the observed GH stimulation of cellular oxygen consumption.

One outstanding question that remains in the field of GH signal transduction is, how are events downstream of GH-induced activation of the GHR/JAK2 complex precisely regulated so that the appropriate response can be achieved in differing cell types and physiological contexts? The ability of adaptor molecules to modulate signal transduction pathways may be one answer to this question. Adaptor proteins lack intrinsic enzymatic activity but generally contain multiple protein-protein interaction domains, serving to recruit additional signaling molecules to the region of activation, allowing for specific activation or modification of those signaling molecules. Additionally, some adaptor proteins can be targeted to different subcellular locations, providing an added level of specificity to the signaling system. The adaptor protein, SH2B1β, was identified as a JAK2 binding protein that is recruited to JAK2 in response to GH (100). Subsequent studies have revealed SH2B1β to be a key modulator of GH signaling (Reviewed in (101)).

SH2B1 in GH Signal Transduction

The SH2B family of adaptor proteins includes SH2B1 (formerly SH2-B for SH2 domain containing protein B, or PSM for proline rich, pleckstrin homology (PH) and SH2 domain-containing signaling modulator), SH2B2 (formerly APS for adaptor protein with PH and SH2 domains) and SH2B3 (formerly Lnk). Each SH2B1 family member was first described as a signaling molecule in immune cell activation (102-104) and each contains a domain structure consisting of a dimerization domain (DD), a PH domain, an SH2 domain and several proline rich regions. These proteins are able to homo and SH2B1 and SH2B2 are able to heterodimerize through their DD domains, presumably allowing for tailored responses to particular signals depending on the relative abundance and subcellular location of SH2B1 and SH2B2. The *SH2B1* message undergoes alternative splicing at the 3' end that results in four SH2B1 proteins (α, β, γ, δ) (105). All four isoforms share an N-terminal DD, nuclear localization sequence (NLS) (106) and nuclear export sequence (NES) (107) as well as C-terminal PH and SH2 domains. The isoforms differ C-terminal to the SH2 domain, and the variation in the C-terminal sequence is known to alter subcellular localization in at least the δ isoform (108). SH2B1 isoforms have been implicated in modulating signals from numerous hormones and growth factors to enhance kinase activity, recruit additional signaling molecules, regulate gene transcription and/or modulate cytoskeletal dynamics (Reviewed in (101)). These multiple functions of SH2B1 allow SH2B1 isoforms to regulate neuronal differentiation (109, 110), energy and glucose homeostasis (111), cell motility (112, 113) and proliferation (105). While it is possible that several SH2B1 isoforms modulate GH signal transduction, the majority of evidence to date has characterized involvement of SH2B1β in GH signaling (Fig. 1.4).

Figure 1.4. Schematic of SH2B1β.
Abbreviations used are DD for dimerization domain, NLS for nuclear localization sequence, NES for nuclear export sequence, PH for pleckstrin homology domain and SH2 for SH2 domain. Tyrosines 439 and 494, which are sites of GH-induced phosphorylation, are represented.

SH2B1β as a JAK2 binding protein

The first indication that SH2B1β might be involved in GH signaling came when the C-terminus of SH2B1β containing the SH2 domain was identified as a binding partner of active, but not kinase-dead, JAK2 in a yeast-2-hybrid assay (100). Subsequent experiments in this study found SH2B1β to co-immunoprecipitate with and be phosphorylated by wild type, but not kinase dead JAK2 when both proteins were exogenously expressed in COS cells. Additionally, Rui et al. found endogenous SH2B1 to co-immunoprecipitate with and be phosphorylated by JAK2 in response GH stimulation of 3T3-F442A cells. Later, SH2B1β was found to enhance JAK2 kinase activity, a function that is dependent on the SH2 domain of SH2B1β, as well as enhance GH-mediated activation of Stat5B (114). Tyrosine (Tyr) 813 of JAK2 was identified as both a GH-induced JAK2 autophosphorylation site and the binding site for the SH2 domain of SH2B1β (12), indicating that GH- or other cytokine-induced phosphorylation of JAK2 is required for SH2 domain-mediated SH2B1β binding to JAK2. Interestingly, a second SH2B1β site that lies outside the SH2 domain was found to be responsible for a

lower-affinity interaction with both active and inactive JAK2 (115), perhaps acting as a "pre-loading" mechanism to facilitate more efficient signaling upon JAK2 activation. Finally JAK2 mediated tyrosyl phosphorylation of SH2B1β was mapped to Tyr439 and 494 (116), both of which were found to be functionally relevant to GH-induced cellular responses (see below).

SH2B1β in GH-induced regulation of the actin cytoskeleton

Some cellular responses to GH stimulation include chondrocyte differentiation (117), monocyte chemotaxis (118), and proliferation and migration of models of epithelial-derived cancer (reviewed in (119)). An essential aspect of each of these responses is a change in cellular morphology, a process that requires regulation of the actin cytoskeleton (Reviewed in (120, 121)), see below).

Regulation of the actin cytoskeleton

The eukaryotic cytoskeleton is a dynamic structure consisting of microfilaments (or actin filaments), intermediate filaments and microtubules. The cytoskeleton is closely linked to the plasma membrane and facilitates cellular motility and division, participates in intracellular transport, and provides structural integrity to give the cell its shape. Actin monomers (G-actin) polymerize to form actin filaments (F-actin), which can then be organized into higher-order functional structures (e.g. stress fibers, filopodia, lamellipodial actin dendritic branches and microspikes). Therefore, regulation of actin polymerization and organization is essential to each of these processes (122). Because intermediate filaments seem to be non-essential for cell shape and microtubules appear to have an indirect (although important) effect on cell shape, it is thought that regulation

of the actin-based cytoskeleton is of principle importance for proper control of the processes mentioned above (reviewed in (123, 124)).

An abundance of proteins are known to bind actin to regulate actin polymerization, depolymerization, organization and localization, resulting in a complex network of regulation (Reviewed in (125)). Actin nucleating proteins, such as Arp2 and Arp3, promote F-actin formation by forming a heterotrimer with G-actin and facilitating rapid growth of the filament (reviewed in (126)). Other proteins, such as the WASP/WAVE family of proteins seem to be necessary for Arp2/3-mediated actin polymerization (reviewed in (127)). Rho GTPases (Rac, Rho and Cdc42) have historically been known to be among the most potent activators of actin polymerization. In recent years, this potent ability to induce formation of F-actin structures such as stress fibers, lamellipodia and filopodia, has been shown to be mediated through Rho GTPase activation of WASP/WAVE proteins. Actin depolymerizing factor (ADF) appears to promote actin depolymerization by binding to the end of F-actin and facilitating dissociation of subunits into G-actin (127). In addition to the regulation of actin-filament dynamics, regulation of higher-order actin filament structures is important for proper cellular function. Actin bundling proteins align F-actin into parallel or anti-parallel linear arrays to form structures such as stress fibers, while actin crosslinking proteins organize F-actin into more perpendicular arrays to form structures such as the actin meshwork that make up lamellipodia (125).

These higher-order actin filaments are tethered to the plasma membrane by a multitude of structural, scaffolding, and transmembrane proteins. Actin stress fibers terminate at focal adhesions, which are integrin-based macromolecular complexes that mediate cell-extracellular matrix (ECM) attachment and facilitate direct signaling between the ECM and the cell (reviewed in (128)) (Fig. 1.5B). Several proteins within focal adhesions bind F-actin, and in this way the cytoskeleton becomes a key

component of cell adhesion sites. In addition to actin-binding proteins, several adaptor, scaffolding and enzymatic proteins are found in focal adhesions. The formation of new adhesion complexes at the leading edge of a cell and the dissolution of focal adhesions complexes at the rear of a cell and the base of cell protrusions (together termed “focal adhesion turnover”) are important events that facilitate cellular motility. Changes in the protein composition of focal adhesions and the activation status of proteins within focal adhesions are two mechanisms by which focal adhesion turnover, and therefore cell motility, is regulated (129).

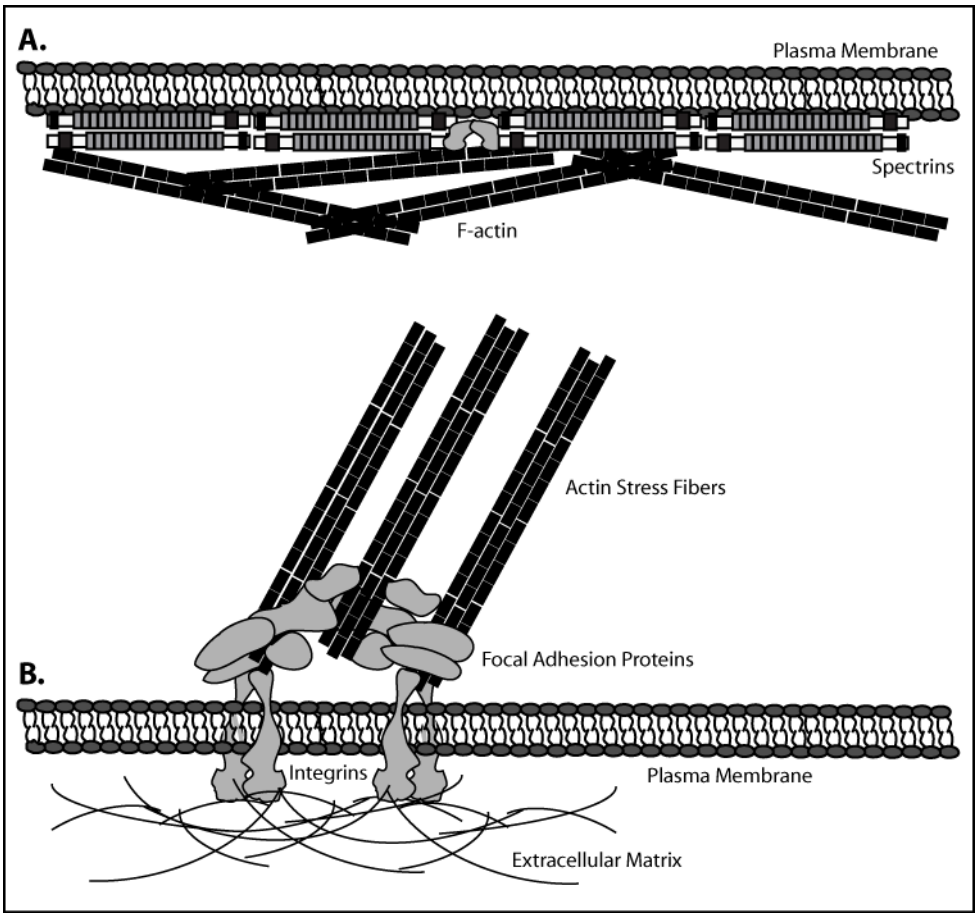

Figure 1.5. Components of the actin cytoskeleton.
(A) Membrane-bound scaffolding proteins such as spectrins form complexes with F-actin to provide structural integrity for a cell. (B) Actin stress fibers are incorporated into integrin-based cell adhesion complexes, connecting the actin cytoskeleton to the extra cellular matrix.

The actin cytoskeleton can also be integrated into the structural framework of the cell by binding scaffolding proteins such as spectrins, which represent a class of actin-binding and crosslinking proteins that also bind the plasma membrane (Figure 1.5A). Many of these proteins, like spectrins, are especially large proteins (> 2000 amino acids) that interact with dozens of other proteins to form a complex network of scaffolding, adaptor and signaling proteins in close association with F-actin and the plasma membrane (reviewed in (130, 131)). Many of these membrane-associated actin-binding proteins are phosphorylated or otherwise modified in response to extracellular and intracellular stimuli. These modifications can alter their localization and/or alter their affinity for F-actin, resulting in attachment or detachment of F-actin to the plasma membrane. Modulation of both actin polymerization dynamics and F-actin attachment to the plasma membrane/cell adhesion sites are the major mechanisms by which cell morphology and motility are regulated.

SH2B1β is required for GH-induced changes in cell morphology

The observation that GH drives processes that require regulation of the actin cytoskeleton, combined with the finding that SH2B1β is required for neuron growth factor (NGF)-induced neurite outgrowth (109, 110)} prompted Herrington et al. (132) to investigate the possibility that SH2B1β is involved in GH-mediated regulation of the actin cytoskeleton. Here, it was demonstrated that SH2B1β co-localizes with actin in GH-induced cell ruffles. This study further showed SH2B1β to enhance both GH- and platelet-derived growth factor (PDGF)-induced ruffling and pinocytosis. However, SH2B1β truncation and point mutants that functionally inactivate the SH2 domain acted as dominant negative proteins in these assays even though GH-induced JAK2 activity was unaffected. These results suggested both that the SH2 domain of SH2B1 is

necessary for ligand-induced cytoskeletal regulation and that a region N-terminal to the SH2 domain interacts with one or more regulators of the actin cytoskeleton. In addition to the SH2 domain, Tyr439/494 were also found to be relevant. Mutation of these Tyr to phenylalanine inhibited GH-induced cell ruffling (116), linking JAK2-mediated phosphorylation of SH2B1β to GH-induced cytoskeletal regulation. These results also raise the possibility that proteins containing SH2 or phosphotyrosine binding domains (PTB) bind SH2B1β to facilitate this function.

The above findings were extended when it was shown that SH2B1β enhances GH-mediated lamellipodia activity and cell migration while SH2 domain mutants again acted as dominant negative proteins (112). In this study, additional C-terminal truncation mutants were found to act in a dominant negative fashion, again suggesting that the N-terminus of SH2B1 interacts with a protein or proteins that regulate the cytoskeleton.

Evidence for the involvement of SH2B1β in cytoskeleton regulation was further demonstrated when it was shown that 1) GH activated endogenous Rac, 2) SH2B1β SH2 domain mutants inhibit constitutively active Rac-induced cell ruffling, and 3) SH2B1β amino acids 85-106 bind Rac (112). As Rac is known to be a major regulator of the cytoskeleton (reviewed in (133)), the results of this study provided one mechanism wherein GH can regulate the cytoskeleton. SH2B1β may recruit Rac into the appropriate location to be activated by GH and/or GH may induce a SH2B1β/active Rac complex to localize to ruffles.

In addition to its involvement in NGF, PDGF and GH-induced responses, SH2B1β is able to cross-link actin filaments *in vitro*, an action that requires two proposed actin-binding domains (a.a. 150-200 and 615-670) (113). In the prokaryotic system, SH2B1β promotes bacterial actin-based motility through an interaction with the bacterial actin binding protein, ActA (a functional homologue of eukaryotic WASP) (134). In

addition to SH2B1, other SH2B family members have been implicated in regulating the cytoskeleton. SH2B2 co-localizes with actin in B cells (135), is thought to modulate actin dynamics in mast cells (136), and interacts with the cytoskeleton regulatory proteins, Vav3 (137) and Enigma (138). SH2B3 is a binding partner of the actin binding and focal adhesion protein, filamin (139). From the above studies, it can be concluded that SH2B1β is an important mediator of GH-induced cytoskeleton regulation, and likely of cytoskeletal regulation in general.

Further actions of GH on the cytoskeleton

In addition to the studies mentioned above, studies specifically investigating GH action on the cytoskeleton found GH stimulation of CHO cells to result in a rapid depolymerization of actin stress fibers, followed by the formation of focal, filamentous, actin-containing complexes (140), as well as alterations in cellular microtubule physiology (141). This latter effect may be due to GH-induced JAK2 phosphorylation of tubulin (142). The Lobie lab has also reported GH to stimulate the formation of a p130cas/CrkII/Src complex that also contains the p85 subunit of PI-3 kinase. They have implicated the formation of this complex in the control of cytoskeletal dynamics (143), as their data indicate that PI-3 kinase regulates GH-stimulated reorganization of the actin cytoskeleton (140) and others have shown that formation of the p130cas-CrkII complex is sufficient for cell migration (144). They also provide evidence for an interaction between JAK2 and FAK (focal adhesion kinase, an important regulator of focal adhesions and cytoskeletal rearrangement) that results in tyrosyl phosphorylation of FAK and two of its focal adhesion substrates, paxillin and tensin (145). More recently, this group has implicated p38 MAPK as being important for GH-induced cytoskeletal rearrangements (146). Interestingly, p130cas, CrkII, Src and FAK are all components of focal adhesions. Mechanistically, as discussed above, the ability of SH2B1β to bind

active Rac seems necessary for GH-induced cell ruffling. It remains to be determined if this interaction is sufficient for all of the processes requiring actin cytoskeleton regulation that SH2B1β has been shown to modulate, or whether SH2B1β interacts with additional components of the cytoskeleton to modulate GH action.

Thesis Summary

Many of the gross events initiated by GH have been delineated. As presented above, the GH-induced activation of JAK2, ERK1/2 and Stat proteins has been well documented across almost all cell and tissue types tested. In addition, many groups have described cellular events such as cytoskeletal reorganization to be a consequence of GH administration. There are also a growing number of studies revealing some of the finer aspects of GH action, such as which genes are transcribed in response to GH-induced Stat activation as opposed to GH-induced ERK1/2 activation (37). Yet, as these aspects of GH action are revealed, further questions arise regarding the specific mechanism of a GH-induced response, along with, how well observations represent GH-induced responses between cell and tissue types. Within the field of GH signaling, there have been an increasing number of reports implicating the Src family kinase proteins as transducers of the GH signal in parallel with or in lieu of JAK2. However, the relative importance of JAK2 and SFKs in two well-established models of GH signaling (3T3-F442A fibroblasts and H4IIE hepatoma cells) had not been assessed. In addition to this question of the primary kinase responsible for GH signaling, the accumulating evidence implicating the downstream signaling molecule, SH2B1β, as a major channel through which GH regulates the cytoskeleton raises the possibility that SH2B1β interacts with several actin-regulating molecules and/or is localized to actin cytoskeletal structures. Therefore, the objective of this thesis work was to 1) determine the relative contribution of JAK2 and Src family kinases to GH-signaling in well-established models of GH signal transduction and 2) more fully characterize the mechanism by which SH2B1β modulates GH-induced regulation of the cytoskeleton.

In Chapter 2, I present data where I, in collaboration with Dr. Hui Jin, utilize pharmacological inhibitors, RNA interference and genetic ablation to investigate the role of JAK2 and Src family kinases to GH signaling in 3T3-F442A and H4IIE cells. Multiple labs have used both cell lines over several years to delineate numerous actions of GH, and therefore, we found it of great importance to determine which kinase(s) were responsible for these actions. We first used antibodies that specifically detect the activated forms of Src family kinases or JAK2 to assess the activation status of these kinases in response to GH. While Src family kinases are active even in the serum-deprived basal state and showed no detectable increase in activation upon GH administration, JAK2 is robustly activated in response to GH in both cell types. To rule out the possibility that increases in Src family kinase activity were below the level of detection by the antibody that we were using or that GH stimulation recruited active SFKs to substrates, we use SFK pharmacological inhibitors to block activation of Src family kinases prior to GH treatment. Compared to control cells, we show that GH is still able to activate ERK1/2 and Stat proteins in the presence of the Src family kinase inhibitors, although we found Akt activation to be decreased compared to control cells. Interestingly, basal Akt activation was also reduced, indicating that the effect of the inhibitors on Akt activation may not be directly related to GH stimulation.

To more fully characterize the contribution of JAK2 to GH signaling in these cells, we created 3T3-F442A and H4IIE cells stably expressing control shRNA or shRNA against JAK2. We found that compared to control cells, JAK2 knockdown cells exhibited an almost complete inhibition of GH activation of Stat3, ERK1/2 and Akt in 3T3-F442A cells and Stat1, Stat5, ERK1/2 and Akt in H4IIE cells. However, although GH-induced Stat5 activation was measurably reduced in knockdown versus control 3T3-F442A cells, the level of reduction is less than the level of JAK2 protein reduction, indicating that some other kinase may be partially responsible for Stat5 activation in 3T3-F442A cells.

To test this possibility, we next assessed Stat5 activation in murine embryonic fibroblasts (MEFs) derived from JAK2 knockout mice. GH treatment of these cells did not result in any detectable activation of Stat5, while WT control MEFs exhibited substantial Stat5 activation. In addition, transient transfection of JAK2 knockout MEFs with JAK2 cDNA rescued the ability of GH to activate Stat5. Taken together, we reasoned that JAK2 is, in fact, the primary kinase responsible for Stat5 activation in these cells. Additionally, we concluded that some signaling molecules are more tightly coupled to the level of JAK2 activation than others (e.g. when JAK2 levels are reduced by 80%, Stat3, ERK1/2 and Akt activation is almost completely abolished while Stat5 activation is reduced to a lesser extent, indicating that lower levels of active JAK2 fully activate Stat5 while full activation of other signaling molecules require higher levels of active JAK2). Finally, we showed that GH-induces activation of Stat3, Stat5, ERK1/2 and Akt in MEFs derived from triple Src family kinase knockout mice, indicating that Src family kinases are dispensable for GH-induced activation of these downstream signaling molecules in these cells. Together, these results indicate that in 3T3-F442A, H4IIE and MEF cells, JAK2, and not Src family kinases, is the primary kinase responsible for Stat, ERK1/2 and Akt activation in response to GH.

In Chapter 3, I further characterized the JAK2-binding protein, SH2B1β, as a mediator of GH-mediated regulation of the actin cytoskeleton. Steven Archer, a former graduate student in the Carter-Su laboratory, showed that amino acids 105-150 of SH2B1β interact with amino acids 2200-2358 of βIIΣ1-spectrin through yeast-2-hybrid and co-immunoprecipitation assays. Because of the perceived importance of SH2B1β to regulation of the actin cytoskeleton, and because spectrins are a major component of the actin cytoskeleton, I sought to further characterize the interaction between SH2B1β and βIIΣ1-spectrin.

βIIΣ1-spectrin (2200-2358) localizes to the cytoplasm and nucleus in 293T cells. SH2B1β exhibits strong plasma membrane and moderate cytoplasmic localization in 293T cells, and SH2B1δ exhibits strong nucleolar localization in 293T cells. Therefore, I first utilized the specific localization patterns of βIIΣ1-spectrin (2200-2358), SH2B1β and SH2B1δ in 293T cells to determine if the SH2B1/βIIΣ1-spectrin interaction was robust enough to alter the localization of βIIΣ1-spectrin (2200-2358). I showed that SH2B1β and SH2B1δ both recruit βIIΣ1-spectrin (2200-2358) to their respective subcellular locations. βIIΣ1-spectrin (2165-2358) localizes to the plasma membrane in 293T cells. SH2B1β (Δ148-198) lacks a plasma membrane binding domain, and thus localizes to the cytoplasm in 293T cells. I further showed that βIIΣ1-spectrin (2165-2358) recruits SH2B1β (Δ148-198) to the plasma membrane. These results suggest that the interaction between SH2B1 and βIIΣ1-spectrin is robust.

I subsequently showed that SH2B1β and full-length βIIΣ1-spectrin co-localize at the plasma membrane with the SH2B1β-binding protein, JAK2, when all three are expressed in 293T cells. Because phosphorylation of spectrins is a major mechanism of regulation of spectrin function, I next sought to determine whether JAK2 phosphorylates βIIΣ1-spectrin. Surprisingly, I found that when both are expressed in 293T cells, JAK2 phosphorylates both βIIΣ1-spectrin and its splice variant, βIIΣ2-spectrin, but does so only in an SH2B1β-dependent manner. To further assess the relationship between these proteins in an endogenous setting and in the context of GH signaling, I turned to a 3T3-F442A fibroblast model in which control shRNA or shRNA against SH2B1β is stably expressed. GH stimulation of control cells resulted in formation of a complex of βII-spectrin, SH2B1β and JAK2, although no GH-induced phosphorylation of βII-spectrin was observed at the 20 min time point tested. However, GH stimulation of SH2B1β

knockdown cells resulted in a significant reduction in the formation of the βII-spectrin/SH2B1/JAK2 complex. These exciting results indicated both that GH treatment induces the formation of a novel complex of βII-spectrin, SH2B1 and JAK2, and that formation of this complex is dependent on SH2B1β. Finally, I demonstrated a possible functional relevance to this relationship by showing that GH treatment of H4IIE cells results in the redistribution of βIIΣ1-spectrin from cell-cell contacts into the cytoplasm. Together, the results suggest that GH stimulation may induce the formation of a βII-spectrin/SH2B1β/JAK2 complex that results in JAK2 phosphorylation of βII-spectrin and βII-spectrin redistribution out of the plasma membrane. βII-spectrin redistribution out of the plasma membrane may contribute to cytoskeleton reorganization, and thus, the data presented in this Chapter identify a component of the actin cytoskeleton as a novel SH2B1β-binding protein and suggest a novel mechanism whereby SH2B1β mediates GH-induced reorganization of the actin cytoskeleton.

In Chapter 4, I characterize an additional novel role of SH2B1β in the actin cytoskeleton. James Herrington, a former post-doctoral fellow in the Carter-Su lab, observed that GFP-tagged WT SH2B1β, but not SH2B1β containing a nonfunctional SH2 domain, localized to focal adhesions in NIH 3T3 cells. I extended these observations in 3T3-F442A cells by showing the SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization. Further, I showed that GH stimulation significantly increases the dynamic cycling of SH2B1β into and out of focal adhesions (focal adhesion protein turnover). I found that stimulation with phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), caused SH2B1β to leave focal adhesions. In contrast, SH2B1β with two serines lying within a PKC substrate consensus motif (serines 161 and 165) mutated to alanine was not induced to leave focal adhesions by PMA stimulation. I expanded these results by showing that the

dynamic turnover of SH2B1β (S161,165A) was significantly reduced and that of SH2B1β (S165E) was slightly increased compared to WT SH2B1β. Finally, I presented evidence suggesting that phosphorylation of serine 165 is functionally relevant by showing that cells expressing SH2B1β (S165E) have significantly more focal adhesions than control cells. Taken together, the results presented in Chapter 4 identify SH2B1β as a novel focal adhesion protein, whose dynamics at focal adhesions are regulated by GH and possibly by PKC-induced phosphorylation of serines 161 and/or 165. In addition, these results suggest that phosphorylation of these serines and subsequent change in SH2B1β dynamics at focal adhesions contributes to the overall number of focal adhesions in cells.

In summary, in this thesis, I demonstrate the primary importance of JAK2 to GH signal transduction in 3T3-F442A and H4IIE cells. Additionally, I characterize βII-spectrin as a novel SH2B1β-interacting protein and provide evidence suggesting that the SH2B1β/βII-spectrin interaction may play a role in GH-induced regulation of the cytoskeleton. Finally, I identify and characterize SH2B1β as a novel focal adhesion protein.

Portions of Chapter 1 are found in my review of GH signaling published in Reviews in Endocrine and Metabolic Disorders (2006). 7:225-2358, under the title, “Recent advances in growth hormone signaling” by Nathan J. Lanning and Christin Carter-Su.

Chapter 2

JAK2, but not Src family kinases, is required for Stat, ERK, and Akt signaling in response to growth hormone in 3T3-F442A preadipocytes and H4IIE hepatoma cells

Abstract

Janus kinase 2 (JAK2), a tyrosine kinase that associates with the growth hormone (GH) receptor and is activated by GH, has been implicated as a key mediator of GH signaling. Several published reports suggest that members of the Src family of tyrosine kinases may also participate in GH signaling. We therefore investigated the extent to which JAK2 and Src family kinases mediate GH activation of STATs 1, 3, and 5a/b, ERKs 1 and 2, and Akt, in the highly GH-responsive cell lines 3T3-F442A preadipocytes and H4IIE hepatoma cells. GH activation of Src family kinases was not detected in either cell line. Further, blocking basal activity of Src kinases with the Src inhibitors PP1 and PP2 did not inhibit GH activation of STATs 1, 3 or 5a/b, or ERKs 1 and 2. When levels of JAK2 were depressed by shRNA in 3T3-F442A and H4IIE cells, GH-stimulated activation of STATs 1, 3 and 5a/b, ERKs 1 and 2, and Akt were significantly reduced, however, basal activity of Src family kinases was unaffected. These results were supported genetically by experiments showing that GH robustly activates JAK2, STATs 3 and 5a/b, ERKs 1 and 2, and Akt in murine embryonic fibroblasts derived from Src/Yes/ Fyn (SYF) triple knock out embryos that lack known Src kinases. These results

strongly suggest that JAK2, but not Src family kinases, is critical for transducing these GH signals in 3T3-F442A and H4IIE cells.

Introduction

Growth hormone (GH) is a peptide hormone that is secreted into the circulation by the anterior pituitary. It is the primary hormone contributing to postnatal body growth (147, 148). It also regulates carbohydrate, fat and protein metabolism (147, 148), immune and cardiac function (149) and aging (150) and has been implicated in cellular proliferation, differentiation, and survival (29). GH signaling pathways are initiated by GH binding to its receptor in the plasma membrane. This binding activates the GH receptor-associated tyrosine kinase Janus kinase 2 (JAK2) which in turn phosphorylates multiple tyrosines within both itself and the GH receptor (5, 25, 151). Multiple signaling molecules have been shown to be recruited to the activated GH receptor-JAK2 complex, leading to the activation of a variety of signaling pathways. Among these pathways are the signal transducer and activator of transcription (STAT), phosphoinositide 3-kinase (PI 3-kinase)/Akt, and MAPK/extracellular regulated kinase (ERK) signaling pathways (25, 29, 36).

Among the seven known mammalian STATs, STATs 1, 3, 5a and 5b have been implicated as GH signaling molecules. In response to GH, these STATs become tyrosyl phosphorylated, dimerize and translocate to the nucleus where they regulate target genes (36, 38). STATs 5a and 5b are thought to be particularly important mediators of GH responses, including body growth, adipose tissue development, and the sexually dimorphic expression of a number of hepatocyte specific genes (42, 152-155).

GH activation of ERKs 1/2 and the PI3-kinase/Akt pathway has been observed both in cell culture (47, 156-160) and in animals (51, 161, 162). Based upon *in vitro* studies using a number of cell types, several different mechanisms have been proposed by which GH activation of JAK2 leads to activation of ERKs 1 and 2. One proposed mechanism involves Shc as the adapter protein linking Grb2 to the activated GH receptor-JAK2 complex, which in turn initiates a Grb2/SOS/Ras/Raf/MEK/ERK1/2 cascade (48-50). GH-induced activation

of ERKs 1 and 2 has also been reported to involve JAK2 phosphorylation of the Grb2 binding site (tyrosine 1068) in the epidermal growth factor receptor and recruitment of Grb2 (51). Others (62, 157, 163) suggest that protein kinase C and/or PI3-kinase activity are required for GH activation of ERKs 1 and 2.

Similarly, several mechanisms for GH activation of the PI3-kinase/Akt pathway have been suggested. One proposed pathway involves JAK2 phosphorylating insulin receptor substrate (IRS) proteins which in turn recruit the p85 subunit of PI3-kinase, thereby activating PI3-kinase (29, 158, 159, 164). Others have shown direct binding of the p85α and β subunits of PI3-kinase to phosphorylated tyrosine residues in the C-terminus of the GH receptor, raising the possibility that GH may promote direct binding of p85 subunits to GH receptor (58).

Although JAK2 is generally believed to be the major tyrosine kinase initiating GH signaling pathways, several studies have suggested that Src family kinases are also capable of binding to the GH receptor and transducing GH signals. There are 8 known members of the mammalian Src kinase family: c-Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, and Blk (165). Like JAK2, c-Src, Yes, and Fyn are expressed in most tissues whereas the other Src family members are expressed predominantly in hematopoietic cells (166). Lck and Lyn are also expressed in neurons (165). Zhu et al. showed that GH could activate Src and Fyn in NIH-3T3 cells (30) and Src in CHO cells ectopically expressing GH receptor (143). Manabe et al. (32) showed that GH can increase Src activity in F-36 human leukemia cells. Based on experiments using the Src family kinase inhibitor, PP2, and antisense c-Src oligonucleotides, Manabe *et al.* also suggest that in F-36 human leukemia cells, Src activates STAT5 in lieu of JAK2. Similarly, Brown et al. (167) report that in FDC-P1 myeloid cells, GH activation of ERKs 1 and 2 is dependent on a Src family kinase. Zhu et al. (30),

using NIH-3T3 cells, also concluded that GH-induced activation of ERKs 1 and 2 is mediated by a JAK2-independent pathway involving c-Src.

In this study, we have examined the relative roles played by endogenous JAK2 and Src family kinases in GH signaling in two well-characterized, GH-responsive cell lines, 3T3-F442A preadipocytes and H4IIE hepatoma cells. GH is required for differentiation of 3T3-F442A preadipocytes into mature adipocytes (168) and regulates the actin cytoskeleton (132, 169). In the differentiated, adipocyte form of 3T3-F442A cells, GH regulates lipolysis, hormone-sensitive lipase (170) and rates of glucose transport (171). It also regulates the transcription of multiple genes, including insulin-like growth factor-1, a number of early response genes, and multiple genes encoding proteins that regulate carbohydrate and lipid metabolism (172). Maximal expression of these genes involves a variety of signaling molecules, including STATs 1, 3 and 5a/b, ERKs 1 and 2, and Akt (5, 28, 37, 47, 156, 157, 160, 173-176). These signaling proteins have also been shown to be activated in H4IIE cells (177-179). H4IIE cells have been used to study the effect of GH on protein synthesis (180) and insulin responsiveness (179, 181, 182).

Using an antibody specific to the activated form of Src family members, we provide evidence that GH does not detectably activate Src family kinases in 3T3-F442A or H4IIE cells. Using Src family kinase inhibitors and shRNA to JAK2 in 3T3-F442A preadipocytes and H4IIE hepatoma cells, and mouse embryo fibroblasts (MEFs) from control, JAK2 knockout, or Src/Yes/Fyn triple knockout mice, we provide strong evidence that GH activation of STATs 1, 3 and 5, ERKs 1 and 2, and Akt are dependent on JAK2 but not Src family kinases. Our studies also reveal that moderate levels of activated JAK2 are sufficient for maximal GH activation of STAT5 in 3T3-F442A cells.

Results

GH does not activate Src family kinases in 3T3-F442A preadipocytes or H4IIE hepatoma cells. As an initial step in investigating whether Src family kinases mediate actions of GH, we examined whether Src family kinases are activated by GH in the GH-responsive 3T3-F442A preadipocyte and H4IIE hepatoma cell lines. 3T3-F442A preadipocytes and H4IIE hepatoma cells were treated with vehicle alone or with GH (500ng/ml) for various times. Lysates from these cells were subjected to SDS-PAGE and subsequent immunoblot analysis with anti-pY416-Src antibody (αpY416-Src), which recognizes the activated form of the Src family members c-Src, Lyn, Fyn, Lck, Yes and Hck. Thus, it would be expected to recognize all forms of Src found in 3T3-F442A and H4IIE cells. As shown in Figs. 2.1A and 2.1B (3rd panel, lane 1), Src family kinases were noticeably active in the basal state in both 3T3-F442A preadipocytes and H4IIE hepatoma cells, respectively. However, GH treatment failed to increase Src family kinase activity above basal levels in either cell line (Figs. 2.1A and 2.1B, 3rd panel, lanes 2-5). Levels of Src family proteins in cell lysates were also unchanged by GH, as judged by immunoblotting cell lysates with anti-Src antibody (αSrc) (Figs. 2.1A and 2.1B, bottom panel).

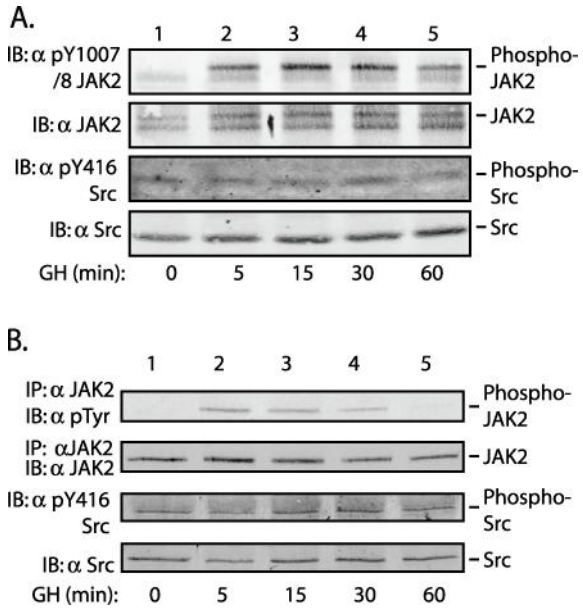

Figure 2.1. GH does not activate Src family member proteins. A) 3T3-F442A preadipocytes were treated with either vehicle for 0 min (lane 1) or with GH (500 ng/ml) for the indicated times (lanes 2-5). Cell lysates were immunoblotted with αpY1007/1008-JAK2 (reprobed with αJAK2) and αpY416-Src (reprobed with αSrc) as indicated (n=3). B) H4IIE hepatoma cells were treated with either vehicle for 0 min (lane 1) or with GH (500 ng/ml) for the indicated times (lanes 2-5). Proteins in an aliquot of H4IIE cell lysates were immunoprecipitated with αJAK2 prior to blotting with αpTyr and reprobing with αJAK2 as indicated. Proteins in aliquots of cell lysates were immunoblotted with αpY416-Src and reprobed with αSrc as indicated (n=4).

To verify that these cells were responsive to GH, the ability of GH to activate JAK2 in 3T3-F442A cells was assessed by blotting lysates with anti-pY1007/1008-JAK2 antibody (αpY1007/1008-JAK2) (Fig. 2.1A, top panel). This antibody recognizes the phosphorylated form of tyrosine(s) 1007 and/or 1008, the activating tyrosines in the kinase domain of JAK2. Phosphorylation of tyrosine 1007 is required for JAK2 activity (15) and generally mirrors overall tyrosyl phosphorylation of JAK2 assessed using an anti-phosphotyrosine antibody (αpY) (12). In contrast to what was observed for Src family kinases, GH caused a rapid and transient phosphorylation of JAK2 on Tyr1007/1008. Phosphorylation of JAK2 was observed as early as 5 min after GH addition, was maximal at 15 and 30 min, and started to

decline within 45 min after GH addition (Fig. 2.1A, top panel). The ability of GH to activate JAK2 in the H4IIE hepatoma cells was assessed by immunoprecipitating JAK2 using αJAK2 and blotting with αpY (Fig. 2.1B, top panel). GH caused a similar rapid and transient tyrosyl phosphorylation of JAK2 in H4IIE cells. A robust signal was evident within 5 min after GH addition, and returned to near basal levels by 60 minutes. Blotting αJAK2 immunoprecipitates with αpY1007/1008-JAK2 revealed a similar time course (see Fig. 2.4B, top panel, lanes 1-5). Immunoblotting cell lysates with αJAK2 (Figs. 2.1A and B, 2nd panels) indicated that endogenous levels of JAK2 were similar for all conditions for both cell types. Taken together, the data in Fig. 2.1 suggest that while GH rapidly and substantially activates JAK2, GH does not appreciably increase total Src family kinase activity in 3T3-F442A preadipocytes or H4IIE hepatoma cells.

Inhibition of Src family kinases does not affect GH activation of JAK2 or tyrosyl phosphorylation of STATs 1, 3 or 5. Multiple Src family members exist in 3T3-F442A preadipocytes and H4IIE hepatoma cells. Because αpY416-Src may detect different Src family members with different affinities and Src family kinases are basally active in these cells, it is possible that we were unable to detect a small GH-induced increase in kinase activity of one or more Src kinase family members that might be important for GH signal transduction. Alternatively, GH might increase Src kinase-substrate interactions by altering the subcellular location of already active Src kinases or the availability of Src kinase substrates. We therefore examined whether Src family inhibitors would inhibit GH activation of JAK2 or the activation of a variety of other GH signaling molecules. 3T3-F442A preadipocytes and H4IIE hepatoma cells were pretreated with vehicle (DMSO), the Src family inhibitors PP1 or PP2, or their inactive analogue, PP3 (183, 184), for 60 min before GH (500 ng/ml) was added for 15 min. As predicted, PP1 and PP2, but not PP3, inhibited Src family kinase activity (assessed using αpY416-Src) in both untreated (Figs. 2.2A and

2.2B, top panel, lanes 1-4) and GH-treated cells (Figs. 2.2A and 2.2B, top panel, lanes 5-8). In contrast, the number of JAK2 proteins activated in response to GH, assessed using αpY1007/1008-JAK2 (Figs. 2.2A and 2.2B, 3rd panel, lanes 5-8) and normalized for the amount of JAK2 (Figs. 2.2A and 2.2B, 4th panel, lanes 5-8) present in each lane, was similar for cells pretreated with vehicle or inhibitors. The lack of a change in JAK2 activation with the Src inhibitors suggests that Src family kinases do not play a role in GH activation of JAK2 in 3T3-F442A preadipocytes or H4IIE hepatoma cells.

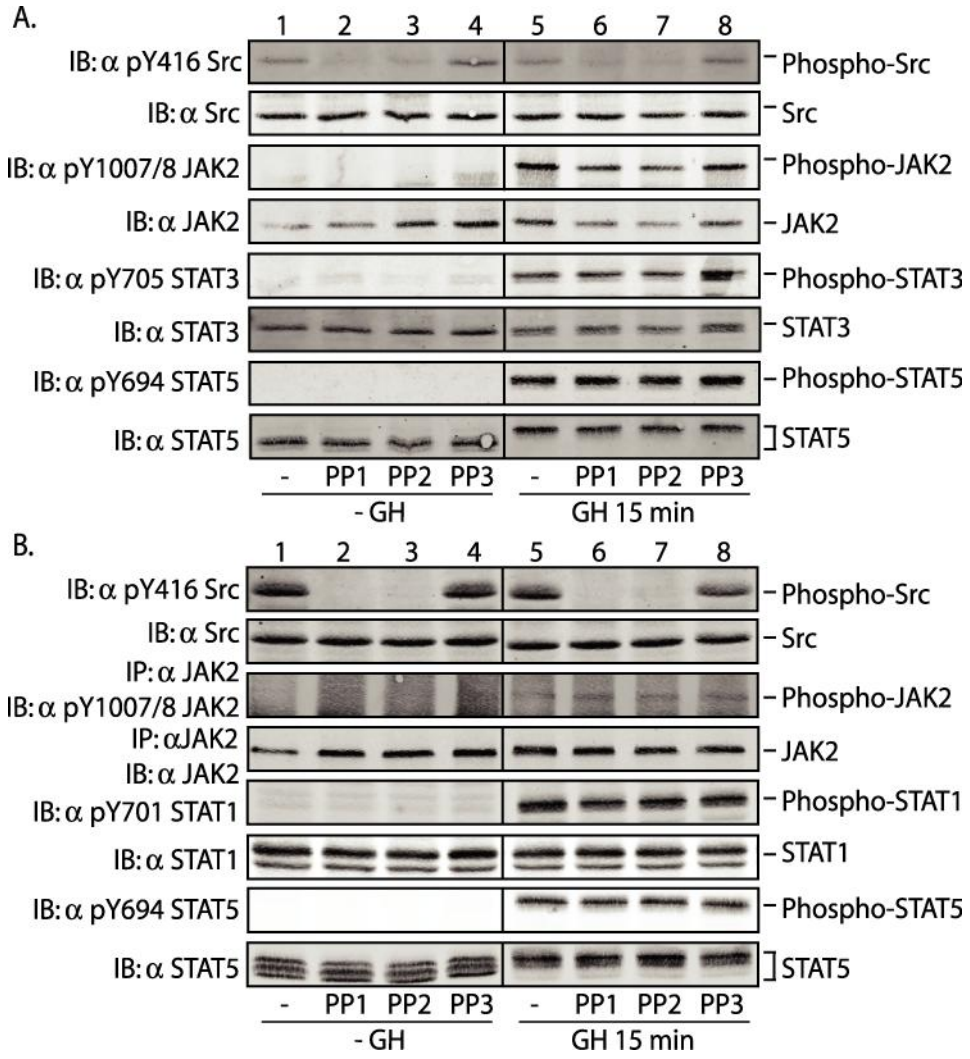

Figure 2.2. GH-stimulated activation of STATs is not blocked by Src kinase inhibitors. A) 3T3-F442A preadipocytes were treated with vehicle (DMSO) (-) or 100 μM PP1, PP2, or PP3 for 60 min before addition of vehicle (-GH) or 500 ng/ml GH (+GH) for 15 min as indicated. Proteins in cell lysates were immunoblotted with αpY416-Src (reprobed with αSrc), αpY1007/1008-JAK2, αJAK2, αpY705-STAT3, αSTAT3, αpY694-STAT5, and αSTAT5 as indicated (n=3). B) H4IIE hepatoma cells were treated with vehicle (DMSO) (-) or 100 μM PP1, PP2, or PP3 for 60 min before addition of vehicle (-GH) or 500 ng/ml GH (+GH) for 15 min as indicated. Proteins in an aliquot of H4IIE cell lysates were immunoprecipitated with αJAK2 prior to blotting with αpY1007/1008-JAK2 and reprobing with αJAK2 as indicated. Proteins in aliquots of cell lysates were immunoblotted with αpY416-Src (reprobed with αSrc), αpY701-STAT1 (reprobed with αSTAT1), αpY694-STAT5, and αSTAT5 as indicated (n=3).

The contribution of Src family kinase activity to GH-activation of STAT proteins was assessed using antibodies that recognize the tyrosyl phosphorylated, activated forms of STAT1 (αpY701-STAT1), STAT3 (αpY705-STAT3) or STATs 5a and 5b (αpY694-STAT5). Phosphorylation of tyrosines 701 (185), 705 (186), and 694/699 (187, 188) is required for activation of STATs 1, 3, and 5a/5b, respectively. Because neither αpY694-STAT5 nor αSTAT5 can distinguish between the very similar STAT5a and STAT5b, we shall use the term STAT5 to indicate both STAT5a and STAT5b. Fig. 2.2A (lanes 5-8) reveals similar levels of GH-stimulated tyrosyl phosphorylation of STAT3 (5th panel) and similar levels of GH-stimulated tyrosyl phosphorylation of STAT5 (7th panel) in 3T3-F442A cells treated with or without the Src family inhibitors PP1 and PP2. PP1 and PP2 also had no effect on the ability of GH to activate STAT1 (Fig. 2.2B, 5th panel, lanes 5-8) or STAT5 in H4IIE cells (Fig. 2.2B, 7th panel, lanes 5-8) or levels of the different STATs in either 3T3-F442A or H4IIE cells (Figs. 2.2A and 2.2B, 6th and bottom panels). Thus, Src family kinases appear not to play a significant mediator role in GH activation of STAT proteins in either 3T3-F442A preadipocytes or H4IIE hepatoma cells.

Effect of Src family inhibitors on GH activation of ERK 1, ERK 2 and Akt. We next examined whether Src family kinases are important for GH activation of ERK1, ERK2 or Akt. Dual phosphorylation of ERK1 on T202 and Y204 and ERK2 on T185 and Y187 (numbering system of human ERKs) is required for their activation. Proteins in aliquots of 3T3-F442A and H4IIE cell lysates from Fig. 2.2 were blotted with an antibody that specifically recognizes ERKs 1 and 2 that are phosphorylated on both the activating Thr and Tyr (αpT202/pY204-ERK1/2). As seen in Fig. 2.3A and 2.3B (top panel, lanes 1 and 5), GH activated ERKs 1 and 2 in 3T3-F442A preadipocytes and H4IIE hepatoma cells. In 3T3-F442A cells, this activation was not reduced when cells were pretreated with PP1, PP2, or PP3 (Fig. 2.3A, top panel, lanes 5-8). In H4IIE cells, PP1 and PP3 reduced GH activated

ERKs 1 and 2, whereas PP2 had no effect (Fig. 2.3B, lanes 5-8, 1st panel). When comparing these results to the results seen in Fig. 2.2B (lanes 5-8, top panel), it becomes apparent that the effect of PP1 on ERK activation in H4IIE cells is not specific to inhibition of Src family kinases. This comparison shows that when Src family kinase activity is undetectable due to pharmacological inhibition by PP2, GH is still able to fully activate ERKs 1 and 2 (compare Fig. 2.2B, lane 7, top panel to Fig. 2.3B, lane 7, top panel). Therefore, the ability of PP1 to inhibit GH-mediated ERK 1 and 2 activation in H4IIE cells cannot be ascribed to a lack of Src family kinase activity. Taking this together with the observation that the negative control (PP3) also significantly inhibits GH-mediated activation of ERKs 1 and 2 but not Src family kinase activity, the conclusion can be drawn that in rat hepatoma cells, PP1 and PP3 inhibit ERK 1 and 2 activation in a non-Src family kinase-specific manner. Thus, these inhibitor studies fail to implicate Src family kinases in GH-mediated activation of ERKs 1 and 2 in 3T3-F442A or H4IIE cells. Whether the PP1/2/3 pattern of inhibition indicates the direct or indirect contribution of some other enzyme to the activation of ERKs 1 and 2 in H4IIE cells but not 3T3-F442A cells is not known. However, the pattern of inhibition does not fit that of tested kinases (189, 190).

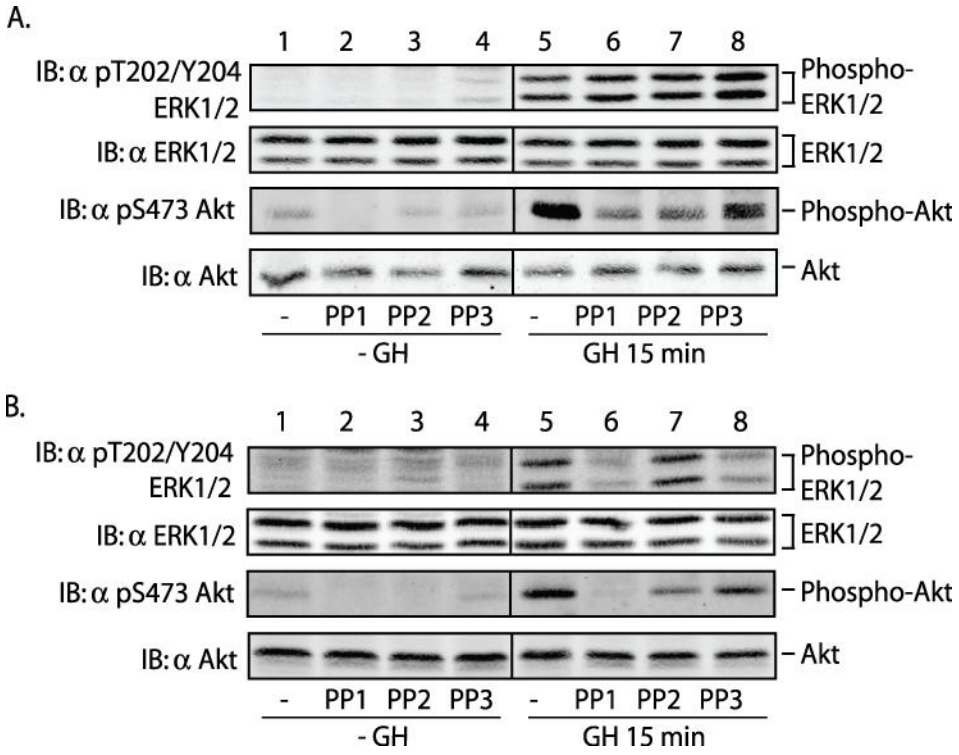

Figure 2.3. Effect of Src kinase inhibitors on GH activation of Erks 1 and 2 and Akt. Proteins in aliquots of cell lysates from 3T3-F442A preadipocytes (A) and H4IIE hepatoma cells (B) used in Figure 2 were immunoblotted with αpT202/pY204-ERK1/2 (reprobed with αERK1/2), αpS473-Akt, and αAkt as indicated (n=3).

Finally, we sought to determine whether Src family kinases are important for GH-stimulated activation of PI3-kinase signaling. Akt, a downstream signaling molecule of PI3-kinase, requires phosphorylation on Ser 473 to be active (191). As shown in Figs. 2.3A and 2.3B (3rd panel, lane 1 vs 5) phosphorylation of Akt on Ser 473 was robust in both 3T3-F442A preadipocytes and H4IIE hepatoma cells stimulated with GH. In both cell types, this level of phosphorylation was substantially reduced in cells pretreated with PP1 and PP2 (Figs. 2.3A and 2.3B, 3rd panel, lanes 5-8). A more modest inhibition was observed in cells pretreated with PP3. The ability of PP1 and PP2 to inhibit phosphorylation of Ser473 in Akt in response to GH raises the possibility that Src family kinases are important for GH activation of Akt. However, PP1 and PP2 (and to a lesser extent PP3) were also found to

reduce basal levels of Akt phosphorylation in both 3T3-F442A and H4IIE cells (Figs. 2.3A and 2.3B, 3rd panel, lanes 1-4), indicating that Src family kinase activity may not mediate GH activation of Akt per sé. Rather, GH activation of Akt may depend on basal priming of Akt by basally active Src. Taken together, the results of Figs. 2 and 3 in which Src family kinases were inhibited in 3T3-F442A preadipocytes and H4IIE hepatoma cells do not support the hypothesis that Src family kinases play a mediator role in the ability of GH to activate JAK2, STATs 1, 3 or 5, or ERKs 1 and 2. However, they do suggest that GH activation of Akt may require basal Src family kinase activity. Because of the inhibition of basal Akt activity by inhibitors of Src family kinases, these experiments are unable to address whether or not Src family kinases play a mediator role in GH activation of Akt.

GH-stimulated activation of STATs 1, 3 and 5, ERKs 1 and 2, and Akt is diminished when endogenous JAK2 levels are reduced in 3T3-F442A preadipocytes and H4IIE hepatoma cells. To determine the degree to which JAK2 is required for GH-mediated activation of STAT proteins, ERKs 1 and 2 and Akt, we examined the ability of GH to activate these signaling molecules when endogenous JAK2 levels were reduced in both 3T3-F442A preadipocytes and H4IIE hepatoma cells. 3T3-F442A preadipocytes and H4IIE hepatoma cells stably expressing control shRNA (Figs. 2.4A and 2.4B, 2nd panel, lanes 1-5) or JAK2 shRNA (Figs. 2.4A and 2.4B, 2nd panel, lanes 6-10) were treated with vehicle or GH (500 ng/ml) for various times. Immunoblotting cell lysates with αJAK2 indicated at least an 80% reduction of endogenous JAK2 protein levels in 3T3-F442A preadipocytes (83% ± 6%, n=3) and H4IIE hepatoma cells (89% ± 4%, n=3) as determined by quantification of JAK2 bands. Immunoblotting with αpY1007/1008-JAK2 confirmed that levels of activated JAK2 are decreased to a similar extent as levels of total JAK2 in the JAK2 shRNA expressing 3T3-F442A and H4IIE cells (Figs. 2.4A and 2.4B, top panels).

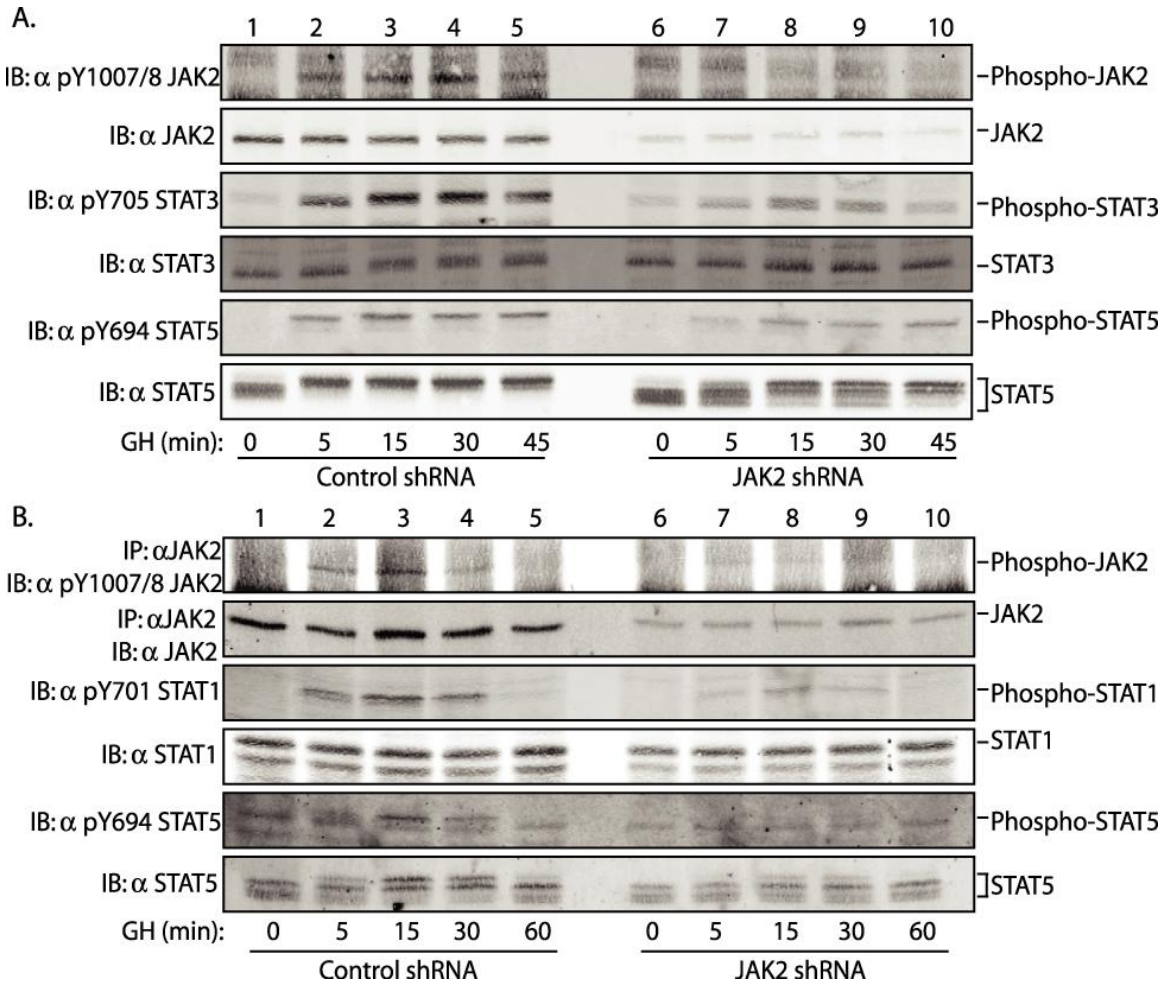

Figure 2.4. GH-mediated STAT activation is significantly diminished when JAK2 levels are reduced using shRNA to JAK2. A) 3T3-F442A preadipocytes stably expressing either control shRNA or JAK2 shRNA were treated with either vehicle for 0 min or with GH (500ng/ml) for the indicated times. Cell lysates were immunoblotted with αpY1007/1008-JAK2, αJAK2, αpY705-STAT3, αSTAT3, αpY694-STAT5, and αSTAT5 as indicated (n=3). B) H4IIE hepatoma cells stably expressing either control shRNA or JAK2 shRNA were treated with either vehicle for 0 min or with GH (500ng/ml) for the indicated times. Proteins in aliquots of H4IIE cell lysates were immunoprecipitated with αJAK2 prior to blotting with αpY1007/1008-JAK2 and reprobing with αJAK2 as indicated. Aliquots of cell lysates were immunoblotted with αpY701-STAT1, αSTAT1, αpY694-STAT5, and αSTAT5 as indicated (n=3).

In control shRNA 3T3-F442A cells, GH-stimulated phosphorylation of STAT3 on Y705 was detectable within 5 min and maximal at 30 min (Fig. 2.4A, 3rd panel, lanes 1-5). GH-stimulated tyrosyl phosphorylation of STAT5 on Y694 was also detectable within 5 min

but remained elevated even after 45 min (Fig. 2.4A, 5th panel, lanes 1-5). Reduction of levels of endogenous JAK2 using shRNA resulted in a substantially reduced GH-dependent phosphorylation of both STAT3 (by 68% ± 4%, n=3) and STAT5 (by 47% ± 7%, n=3) (Fig. 2.4A, 3rd and 5th panels, respectively). Reduction of endogenous JAK2 did not alter levels of STAT3 or STAT5 (Fig. 2.4A, 4th and 6th panels, respectively). In control shRNA expressing H4IIE cells, GH-stimulated phosphorylation of both STAT1 on Y701 and STAT5 on Y694 was detectable within 5 min, was maximal at 15 min and returned to near basal values by 60 min. In the shRNA-JAK2 H4IIE cells, GH-dependent phosphorylation of both STAT1 and STAT5 was substantially reduced at all time points (Fig. 2.4B, 3rd and 5th panels respectively). Reduction of endogenous JAK2 did not alter levels of STAT1 or STAT5 (Fig. 2.4B, 4th and 6th panels, respectively). Thus, reduction of endogenous JAK2 substantially reduces the ability of GH to activate STAT proteins in both 3T3-F442A preadipocytes and H4IIE hepatoma cells.

We next determined the importance of JAK2 for GH-mediated activation of ERKs 1 and 2. GH stimulation of control shRNA 3T3-F442A preadipocytes resulted in the transient activation of ERKs 1 and 2, which was evident within 5 min and over by 45 min (Fig. 2.5A, top panel, lanes 1-5). Activation of ERKs 1 and 2 was almost eliminated in cells expressing JAK2 shRNA, being detectable above basal values only at the 5 min time point (Fig. 2.5A, top panel, lanes 6-10). GH stimulation of control shRNA H4IIE hepatoma cells resulted in a relatively modest increase in activation of ERKs 1 and 2, visible in Fig. 2.5B (top panel, lanes 1-5) at the 15 and 30 min time points. No GH stimulation of ERKs 1 and 2 was detectable in the JAK2 shRNA hepatoma cells (Fig. 2.5B, top panel, lanes 6-10). Reduction of endogenous JAK2 did not alter levels of ERKs 1 or 2 in either cell line (Fig. 2.5A and 2.5B, 2nd panels). Thus, reduction of endogenous JAK2 substantially reduces the ability of GH to activate ERKs 1 and 2 in both 3T3-F442A preadipocytes and H4IIE hepatoma cells.

For Akt, GH (500 ng/ml) caused a robust increase in phosphorylation of Ser473 within 5 min in both control shRNA 3T3-F442A preadipocytes and H4IIE hepatoma cells (Figs. 2.5A and 2.5B, 3rd panels, lanes 1-5). Phosphorylation was sustained for 30 min in the 3T3-F442A cells before declining whereas it started declining within 15 min in the H4IIE cells. For both cell types, however, phosphorylation of Ser473 was reduced to barely detectable values at all time points by the reduction of JAK2 with shRNA JAK2 (Figs. 2.5A and 2.5B, 3rd panels, lanes 6-10). Akt levels were not similarly reduced in either control or shRNA JAK2 expressing cells (Figs. 2.5A and 2.5B, 4th panels). The fact that reducing JAK2 is so effective in reducing levels of GH activation of Akt supports the hypothesis that JAK2 is the primary kinase responsible for GH activation of the PI3 kinase-Akt pathway.

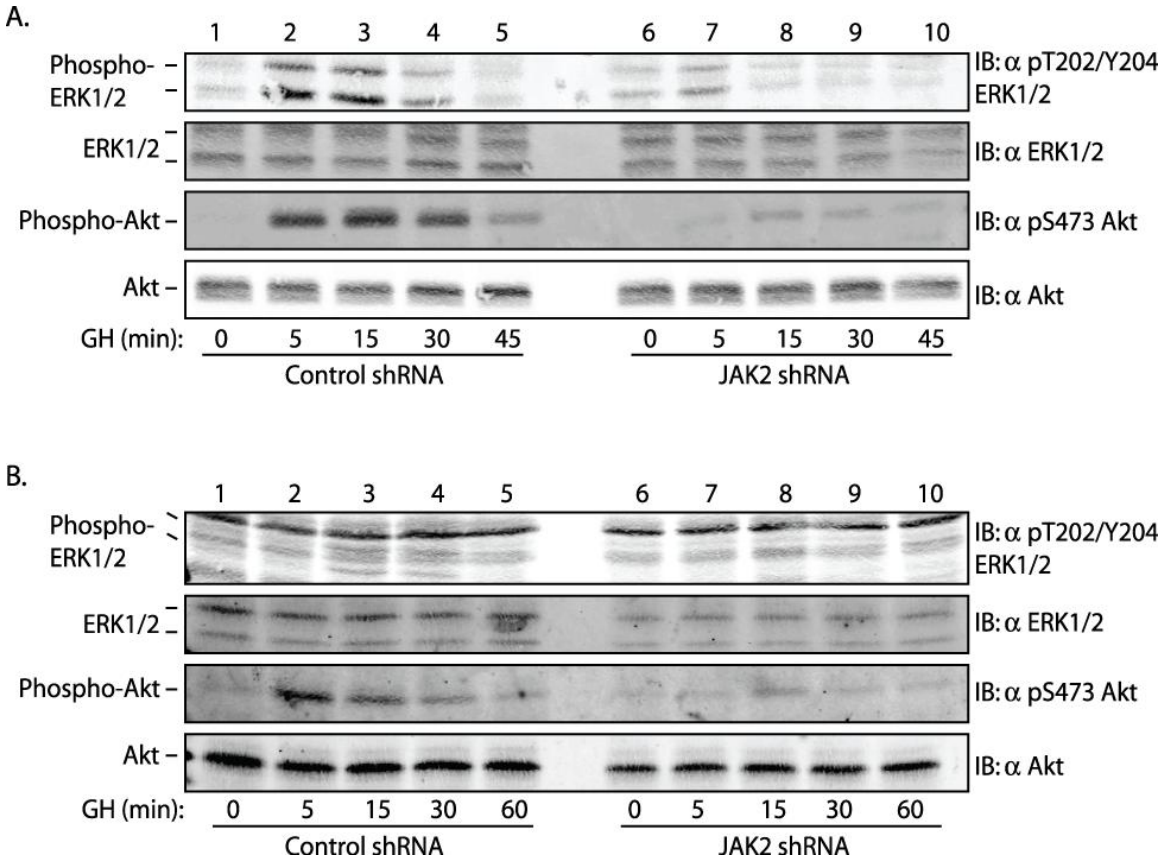

Figure 2.5. GH activation of Erk 1, Erk 2 and Akt is diminished substantially when JAK2 levels are reduced using shRNA to JAK2. Proteins in aliquots of the lysates of (A) 3T3-F442A preadipocytes stably expressing either control shRNA or JAK2 shRNA or (B) H4IIE hepatoma cells stably expressing either control shRNA or JAK2 shRNA used in Figure 4 were immunoblotted with αpT202/pY204-ERK1/2, αERK1/2, αpS473-Akt, and αAkt as indicated (n=3).

To rule out the possibility that reduction of endogenous JAK2 reduces the level of Src family kinases, lysates from both 3T3-F442A and H4IIE cells were blotted with αpY416-Src and αSrc (Figs. 2.6A and 2.6B, panels 3 and 4). No differences in levels of Src activation or Src protein were observed between control and JAK2 knock down cells, indicating that reducing the level of JAK2 does not affect basal Src family kinase activity. Thus, basal Src family kinase activity appears to be independent of JAK2. To test whether

the decreased responsiveness to GH of the JAK2 shRNA cells compared to the control shRNA cells could be a result of reduced expression of the GH receptor, control and shRNA expressing 3T3-F442A cells were treated with GH for 15 min. GH receptor levels were similar in control and JAK2 shRNA expressing cells as shown by blotting lysates with antibody to the intracellular domain of the GH receptor (Fig. 2.6C middle panel). When GH receptor was immunoprecipitated using antibody to the extracellular domain of the GH receptor and blotted with αPY, tyrosyl phosphorylation of GH receptor was found to be reduced in JAK2 shRNA cells (Fig. 2.6C, bottom panel) compared to control shRNA cells, as one would predict from the decreased levels of JAK2. Taken together, the data of Figs. 2.4-2.6 indicate that JAK2 is required for maximal GH-mediated activation of STATs 1, 3 and 5, ERKs 1 and 2, and Akt. In contrast, Src family kinase activity is independent of JAK2.

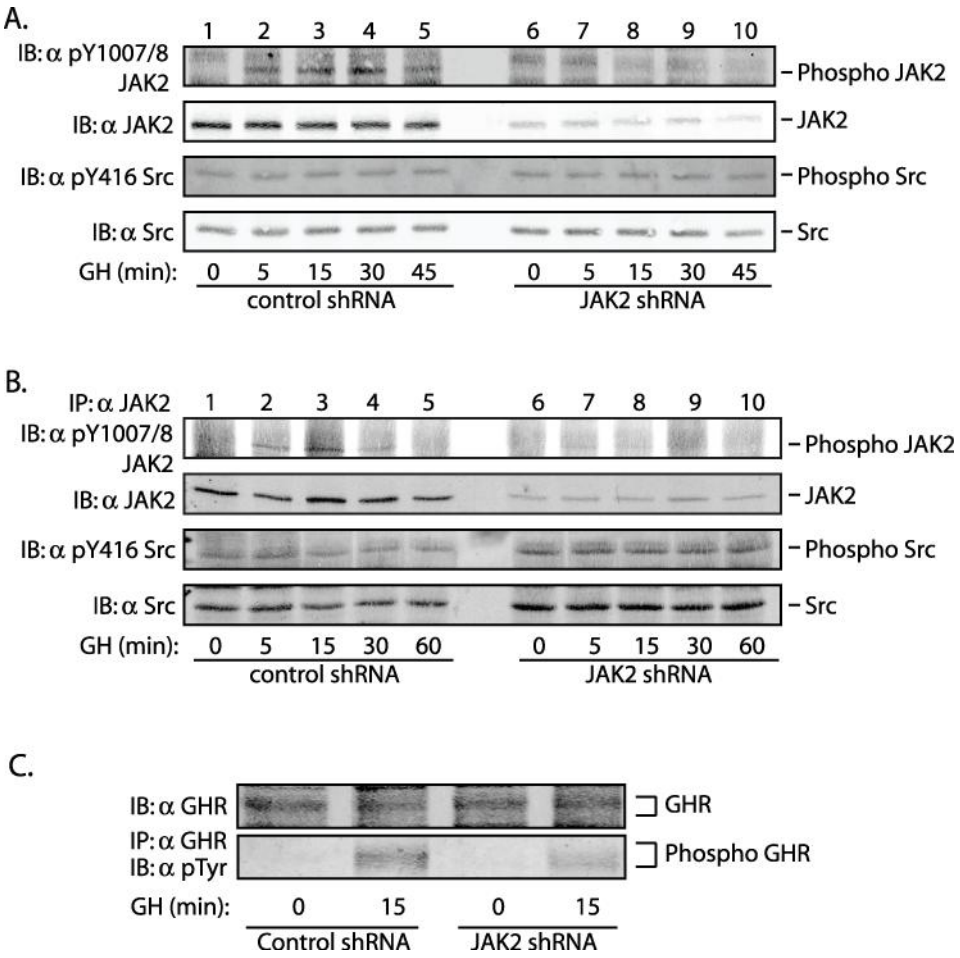

Figure 2.6. Src family kinase activity is not affected by reducing levels of JAK2. A) Proteins in aliquots of the lysates from 3T3-F442A preadipocytes stably expressing either control shRNA or JAK2 shRNA used in Figure 4 were immunoblotted with αpY1007/1008-JAK2, αJAK2, and αpY416-Src (reprobed with αSrc) as indicated (n=3). B) Proteins in aliquots of the lysates from H4IIE hepatoma cells stably expressing either control shRNA or JAK2 shRNA used in Figure 4 were immunoprecipitated with αJAK2 prior to blotting with αpY1007/1008-JAK2 and reprobing with αJAK2 as indicated (n=3). Proteins in aliquots of cell lysates were immunoblotted with αpY416-Src and reprobed with αSrc as indicated (n=3). The top 2 panels for (A) and (B) are the same as the top 2 panels in Figs. 4A and 4B, respectively. C) 3T3-F442A preadipocytes stably expressing either control shRNA or JAK2 shRNA were treated with either vehicle for 0 min or GH (500ng/ml) for the indicated time. Aliquots were immunoprecipitated with αGHBP prior to blotting with αpTyr. Aliquots of cell lysates were immunoblotted with αGHR (AL47) as indicated (n=3).

GH activates JAK2, STATs 1, 3 and 5, ERKs 1 and 2, and Akt in cells lacking Src family members. To provide genetic evidence that JAK2, but not Src family kinases, plays a direct and essential role in GH-induced activation of signaling molecules, mouse embryo fibroblasts (MEFs) derived from embryos from Src/yes/fyn (SYF) triple knock out mice (192) were tested for their responses to GH. SYF MEF cells also lack Lyn expression. Thus, these cells lack all Src family members reported to be present in murine fibroblasts (192). This absence of Src family members was confirmed by the absence of any detectable signal in αSrc or αpY416 Src immunoblots of SYF MEF cells (Fig. 2.7, top 2 panels, compare to wild-type MEFs shown in Fig. 2.8A, panels 5 and 6, lanes 1-5). SYF MEFs were treated with vehicle alone or with GH (500 ng/ml) for various times. Immunoblots of lysates from these cells revealed that JAK2, STAT3, STAT5, ERK1, ERK2 and Akt are all robustly phosphorylated in response to GH (Fig. 2.7). These results provide strong evidence that Src family kinases are dispensable for GH activation of these signaling proteins.

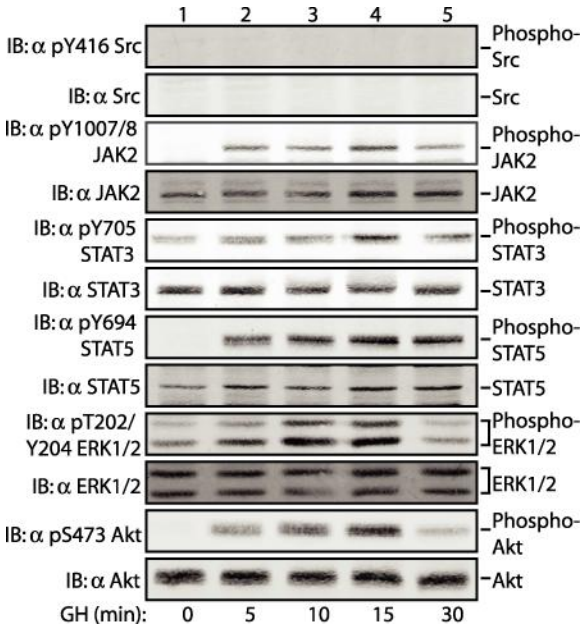

Figure 2.7. GH activates JAK2, STATs 1, 3 and 5, Erks 1 and 2, and Akt in SYF MEF cells. SYF MEF cells were treated with either vehicle (0 min) or with GH (500ng/ml) for the indicated time. Proteins in cell lysates were blotted with αpY416-Src (reprobed with αSrc), αpY1007/1008-JAK2 (reprobed with αJAK2), αpY705-STAT3, αSTAT3, αpY694-STAT5, αSTAT5, αpT202/Y204-ERK1/2 (reprobed with αErk1/2), αpS473-Akt, and αAkt as indicated (n=3).

GH is unable to activate STAT5 in JAK2$^{-/-}$ MEFs. The finding that in shRNA-JAK2 3T3-F442A cells, GH stimulation of STAT5 phosphorylation was reduced to a lesser extent than levels of JAK2 (Fig. 2.4A) allows for the possibility that some other kinase contributes to that stimulation. The SYF MEF and Src family inhibitor studies suggest members of the Src family of tyrosine kinases do not contribute. However, those results do not allow us to rule out another, unidentified kinase. To test the possibility that some kinase other than JAK2 mediates GH activation of STAT5, we examined to what degree deleting all JAK2 would block GH activation of STAT5. Wild-type and JAK2$^{-/-}$ MEFs were treated with vehicle alone or with GH (500 ng/ml) for various times. Immunoblotting cell lysates with αJAK2 confirmed the complete loss of JAK2 expression in the JAK2$^{-/-}$ MEF cells (Fig. 2.8A, 2nd panel). As predicted, blotting the lysates with αpY1007/1008-JAK2 showed that, similar to

what we observed in 3T3-F442A and HEII4 cells, JAK2 was rapidly and transiently phosphorylated on Tyr1007/1008 in wild-type MEFs while no phosphorylated JAK2 was detectable in JAK2$^{-/-}$ MEFs (Fig 2.8A, top panel). Similar to what we observed with lysates from SYF MEF cells, immunoblots of the wild-type MEF lysates with αpY694-STAT5 revealed a robust activation of STAT5 that was evident at 5 min and maximal at 10-15 min (Fig. 2.8A, lanes 1-5, 3rd panel). However, there was no detectable phosphorylated STAT5 in the lysate from JAK2$^{-/-}$ MEFs (Fig. 2.8A, lanes 6-10, 3rd panel), suggesting a total dependency on JAK2 of STAT5 activation. Blotting the lysates with αpY416-Src showed no difference in Src kinase phosphorylation following GH addition or between the wild-type and JAK2$^{-/-}$ MEFs (Fig. 2.8A, panels 5 and 6). Levels of STAT5 and Src were similar in wild-type and JAK2$^{-/-}$ MEFs (Fig. 2.8A, panels 4 and 6). Therefore, the loss of activation of STAT5 in JAK2$^{-/-}$ cells is most likely due to the lack of JAK2, and not indirectly to changes in the activity of Src family kinase activity or any other kinases. Reintroducing wild-type JAK2 into JAK2$^{-/-}$ MEFs rescued GH-stimulated STAT5 activation, to a level commensurate with the level of reintroduced JAK2 (Fig. 2.8B), providing further support for the hypothesis that JAK2 is required for GH activation of STAT5.

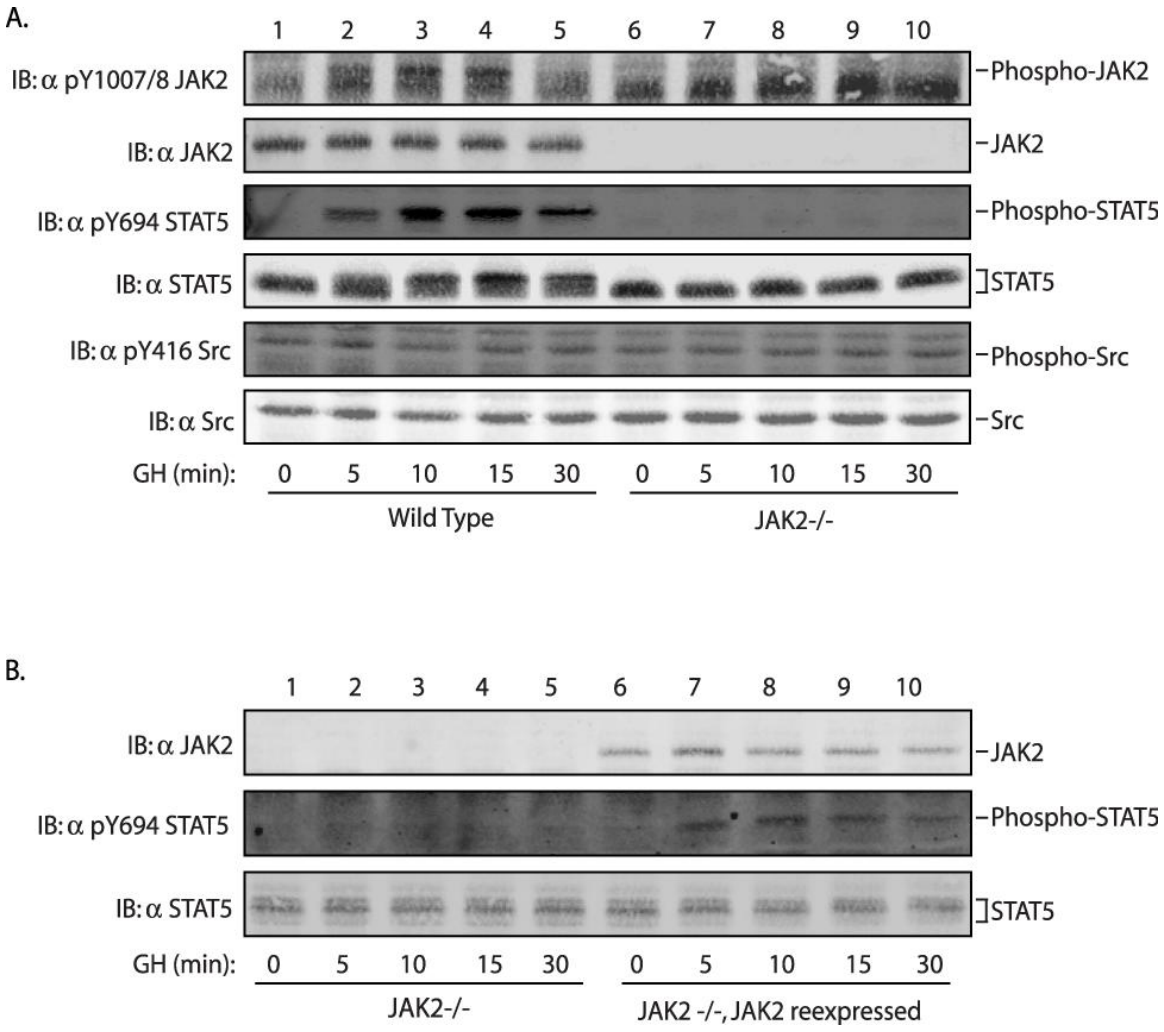

Figure 2.8. GH is unable to activate STAT5 in MEFs lacking JAK2. A) Wild-type MEFs or MEFs from JAK2$^{-/-}$ mice were treated with either vehicle (0 min) or with GH (500ng/ml) for the indicated times. Cell lysates were immunoblotted with αpY1007/1008-JAK2 (reprobed withαJAK2), αpY694-STAT5, αSTAT5, and αpY416-Src (reprobed with αSrc) as indicated (n=3). B) MEFs from JAK2$^{-/-}$ mice or the same MEFs in which JAK2 was stably reintroduced were treated with either vehicle (0 min) or with GH (500ng/ml) for the indicated times. Cell lysates were immunoblotted with either αJAK2, αpY694-STAT5, or αSTAT5 as indicated (n=2).

DISCUSSION

Because members of the cytokine family of receptors do not have intrinsic kinase activity, the signaling events initiated by cytokines are achieved through activation of receptor-associated tyrosine kinases, primarily members of the JAK family of tyrosine kinases (reviewed in (193)). However, some cytokine receptors have been reported to bind to and activate members of the Src family of tyrosine kinases (165). In the case of GH, JAK2 has classically been thought to be the major kinase responsible for initiating downstream signaling events although JAK1 and JAK3 have been shown to be minimally activated in some cells (26, 194). Multiple studies have demonstrated an interaction between GH receptor and JAK2 and a robust activation of JAK2 following GH binding of GH receptor (4, 5, 25). However, several studies have suggested that GH may also activate members of the Src family of tyrosine kinases and that activation of Src family members may contribute to activation of signaling molecules downstream of GH, including STAT5 and ERKs 1 and 2 (30, 32).

In this study, we sought to determine the relative contribution of Src family kinases to GH signaling by assessing the activation status of endogenous GH signaling proteins in cell lines that have been well characterized for GH signaling and GH responses. Using an antibody to the phosphorylated form of the activating tyrosine in JAK2 to assess levels of activated JAK2, we found that JAK2 was inactive in both 3T3-F442A and H4IIE cells that had not seen GH. Upon GH treatment, JAK2 was rapidly and transiently activated, as reported previously (5, 177). In contrast, using an antibody that recognizes the phosphorylated form of the activating tyrosine in Src family kinases to assess levels of activated Src family kinases, we found that Src kinases are basally active in 3T3-F442A and H4IIE cells. GH treatment did not appreciably enhance that activity at early time points (30 sec and 2 min) (data not shown) or over an extended period of treatment (up to 60 min) (Fig.

2.1). Thus, it seems unlikely that in these cell types, Src kinases can substitute for JAK2 as important mediators of GH action, unless GH activates only a small, undetectable, subset of the Src family kinases or increases Src kinase-substrate interactions (e.g. by altering the subcellular location of already active Src kinases or the availability of Src kinase substrates). Similarly, Yamauchi et al. (51) reported seeing no GH-induced increase in Src activity. The reason why these results differ from those of the groups observing a GH-induced increase in the activity of Src family kinases is not clear. Possible explanations include differences in culture conditions or cell type. Relevant to the former, we observed no GH-induced increase in Src kinase activity in either subconfluent (70-80%) or confluent 3T3-F442A cells (data not shown). Regarding the latter, all the different groups used different cell types. Zhu et al. reported GH induced activity of Src kinases in CHO cells overexpressing GH receptor (c-Src and c-Fyn) (143) and in NIH-3T3 cells (c-Src) (30) while Manabe et al. (32) and Brown et al. (167) reported GH induction of Src using F-36P human leukemia cells (c-Src) and FDC-P1 myeloid cells (Lyn), respectively. Circulating cells, such as the F-36P and FDC-P1 cells, in general seem to have a greater propensity for utilizing Src family kinases for cytokine signaling compared to non-circulating cell types (165), raising the question of whether the ratio of Src family kinases to JAK2 is higher in these cells or they have accessory proteins that enable or enhance cytokine activation of Src kinases. It is interesting to note that even when GH was observed to activate Src family kinases, the degree of stimulation when assessed quantitatively, was quite modest, between 30-70% (143), in contrast to the degree of GH stimulation of JAK2 that generally shows a robust on/off type of response. It is also important to note that we found the activity of Src family kinases to be unaffected by the level of JAK2 and vice-versa. Thus, reducing levels of JAK2 in 3T3-F442A and H4IIE cells by shRNA to JAK2 or in MEFs genetically deleted for JAK2 did not decrease the level of activity of Src kinases, nor did reducing the activity of Src family kinases using PP1 and PP2 alter the ability of GH to activate JAK2. This independence of

Src and JAK2 activity supports the previous findings of Zhu et al. (30) in NIH-3T3 cells using both pharmacological inhibitors (PP1, PP2 and AG490) and dominant negative constructs of Src and JAK2. It also argues against Src being recruited to GH receptor-JAK2 complexes and being activated as a consequence of binding to tyrosines within JAK2 or GH receptor that are phosphorylated by JAK2 in response to GH.

The fact that GH did not appear to activate Src family kinases in our experiments does not a priori exclude them from being mediators of GH signaling, since it is possible that GH elicits a small, undetectable increase in the activity of one of more Src kinases, alters the subcellular location of already active Src kinases or alters the availability of Src kinase substrates. However, our data using the Src family kinase inhibitors PP1 and PP2 reveal that blocking the activity of Src family kinases in 3T3-F442A preadipocytes and H4IIE hepatoma cells does not attenuate GH-mediated activation of STATs 1, 3 or 5, indicating that activation of these signaling molecules by GH is independent of Src in these cells. The inability of PP1 and PP2 to block GH activation of STAT5 is consistent with the previous report of Guren et al. (195) showing no reduction in cultured rat hepatocytes of GH-mediated STAT5 activation by a different Src kinase inhibitor, CGP77675. It is also consistent with the finding that STAT5 is activated by GH in CHO cells stably expressing wild-type GH receptor but not in CHO cells stably expressing a mutated GH receptor lacking the binding site for JAK2 (28). However, it contrasts with the finding of Manabe et al. (32), who showed a PP2-dependent, Src antisense oligonucleotide-sensitive, inhibition of GH-mediated STAT5 phosphorylation in F-36P cells. One explanation for the apparent discrepancy between these studies is a difference in cell type, with fibroblasts, preadipocytes and hepatocytes relying solely on JAK2 for GH activation of STAT5 and circulating cells being able to utilize Src family kinases in addition to or in place of JAK2. Unfortunately, in the latter study, the authors did not explore the relative contributions of JAK2 and Src kinases to the GH activation of STAT5, so that it is unclear whether in F-36P

cells, Src family kinases mediate or modulate GH activation of STAT5, and whether that action is independent of JAK2. In further support of JAK2 and not Src kinases being responsible for GH activation of STAT5, we observed a robust activation of STAT5 by GH in SYF MEFs that are genetically deleted for SFKs and the absence of STAT5 activation in MEFs genetically deleted for JAK2. The latter was rescued upon reintroduction of JAK2. GH activation of STAT5 was also significantly decreased in 3T3-F442A and H4IIE cells with reduced levels of JAK2 due to expression of shRNA to JAK2.

Similar to our results with STAT5, our findings with STATS 1 and 3 suggest that their activation by GH is highly dependent upon JAK2 and independent of Src family kinases. The independence from Src family kinases is supported by the findings that PP1 and PP2 eliminated Src activity but had no effect on the ability of GH to activate STATs 1 and 3 in H4IIE and 3T3-F442A cells, respectively. In addition, STAT3 was robustly activated by GH in SYF MEFs that are genetically deleted for SFKs. In support of their activation being dependent upon JAK2, GH activation of STATs 1 and 3 above basal values was severely depressed in H4IIE and 3T3-F442A cells, respectively, in which JAK2 levels were reduced using shRNA to JAK2. The dependence of GH activation of STATs 1 and 3 on JAK2 is consistent with the finding of Han et al. (196) that STATs 1 and 3 are activated by GH in wild-type H1080 cells but not in H1080 cells lacking intact JAK2.

Our Src family kinase chemical inhibitor and SYF MEF experiments also demonstrate that GH-mediated activation of ERKs 1 and 2 is not dependant on Src family kinases in 3T3-F442A, H4IIE or MEF cells. This finding is consistent with the previous findings that Shc phosphorylation and MAP kinase activity are stimulated by GH in CHO-GH receptor cells but not in CHO cells stably expressing a mutated GH receptor lacking the binding site for JAK2 (48, 197). It is also consistent with the report that Shc phosphorylation is stimulated by GH in wild-type H1080 cells but not in H1080 cells lacking JAK2 (196). Zhu et al. (30, 31) proposed a JAK-independent, Src-dependent mechanism for activation of

ERKs 1 and 2 based on the observations that GH activates c-Src (as well as JAK2) in NIH-3T3 cells, GH stimulates Ral A and Ral B, GH-activated RalA results in an increase in phospholipase D activity and the production of phosphatidic acid, and RalA, phospholipase D activity and phosphatidic acid are all required for GH-stimulated activation of ERKs 1 and 2 as assessed using an Elk-1 reporter assay. However, this group did not actually test directly the effects of decreasing levels of JAK2 or Src family kinase activity (by use of pharmacological inhibitors or decreasing levels of expression) on the ability of GH to activate ERKs 1 and 2. Thus, the relative contributions of JAK2 and Src kinases to GH activation of ERKs 1 and 2 are not clear, nor is it clear from those studies whether Src is sufficient, or simply necessary, for GH activation of ERKs 1 and 2. Finally, Gu et al. (198) raise the possibility of Src regulating GH-mediated activation of ERK2 by showing that overexpression of Csk (a protein that inactivates Src family kinases) in cardiac myocytes inhibits the ability of GH to activate overexpressed ERK2. Unfortunately, Src family kinase and JAK2 activities were not assessed in the context of Csk overexpression, raising the possibility that this effect of Csk overexpression was not Src family kinase-specific. Furthermore, inhibitors of JAK2, EGF receptor and Src all blocked GH stimulation of ERKs 1 and 2 in these cells, confounding the assessment of the role of Src kinases in the process.

In the case of Akt, we observed in both 3T3-F442A and H4IIE cells a PP1 and PP2-dependent inhibition of GH-mediated phosphorylation on Ser473, raising the possibility that GH activation of Akt may require Src family kinases. This would be consistent with studies in human neutrophils and BAF3 cells that suggest that the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) may signal to STATs and MAP kinases through JAKs but signal to Akt through Src family kinases (199, 200). However, we found that GH robustly stimulates the phosphorylation of Ser-473 in Akt in MEFs genetically deleted for Src family kinases. Furthermore, reduction of endogenous JAK2 levels by shRNA reduced GH-mediated Akt

activation to barely detectable levels in both 3T3-F442A and H4IIE cells, indicating that JAK2 is essential for GH-mediated Akt activation in these cells. Consistent with Akt activation requiring JAK2, Yamauchi et al. (161) found in γ2A-GHR cells lacking JAK2, that GH is unable to stimulate the tyrosyl phosphorylation of IRS-1, IRS-2, and IRS-3, their association with p85 subunit of PI3-kinase and the activation of PI3-kinase, events that are thought to link GH receptor to Akt activation. Similarly, Argetsinger et al. (158, 164) found that GH stimulated tyrosyl phosphorylation of IRS 1 and 2 in CHO-GHR cells but not in CHO cells stably expressing a mutated GH receptor lacking the binding site for JAK2. Thus, our finding that PP1 and PP2 inhibit GH-induced Ser473 phosphorylation of Akt raises the possibility that Src activity, rather than being a necessary component linking GH receptor to Akt, may be indirectly required for GH to activate Akt. Supporting this hypothesis, the Qui group (201, 202) has described a potential mechanism whereby Src must phosphorylate Akt on Tyr315 and Tyr326 prior to growth factor-dependent phosphorylation of Thr308 and Ser473. Consistent with this, our data show that both PP1 and PP2 inhibit basally active Akt (Fig. 2.3 A and B), raising the possibility that maximal phosphorylation of Akt Ser473 by any factor is unachievable when Src activity is decreased. In support of this idea, we found that EGF is also unable to stimulate phosphorylation of Ser473 in Akt when 3T3-F442A preadipocytes are pretreated with PP1 or PP2 (data not shown). Because PP3 at the concentrations used did not inhibit Src kinase activity, the finding that PP3 inhibits both basal and GH stimulated phosphorylation of Ser473 in Akt also raises the possibility that the effects of PP1 and PP2 are not mediated exclusively via Src family kinases. PP1 and PP2 have been reported to have significant off-target effects (189, 190).

An interesting byproduct of our studies is the observation that some signaling pathways are more tightly coupled to the level of activation of JAK2 than others. Thus, when JAK2 levels were reduced by ~80% by shRNA against JAK2 in 3T3-F442A preadipocytes, the ability of GH to activate JAK2, ERKs 1 and 2, Akt, and STAT3 was

reduced to a similar extent. In contrast, the ability of GH to stimulate the tyrosyl phosphorylation of STAT5 was reduced by only ~50%. In JAK2 shRNA 3T3-F442A cells that exhibited only a 50-60% reduction of JAK2 (as quantified from all time points in two independent experiments), GH activation of ERKs 1 and 2 and Akt was again almost abolished, whereas GH stimulation of STAT5 activity was relatively unaffected (data not shown). Although one could argue that this apparent discrepancy is because another kinase is necessary for maximal GH activation of STAT5, the MEF data argue that JAK2 is required for GH activation of STAT5 since we detected no GH stimulation of STAT5 when JAK2 was completely absent. These results therefore suggest that in the case of ERKs 1 and 2, Akt and STAT3, levels of activated JAK2 are rate-limiting, whereas they are not for STAT5. The MEF data also show that replacement of only a small amount of JAK2 is able to reconstitute substantial GH activation of STAT5. That levels of STAT5 rather than levels of JAK2 appear to be rate-limiting in 3T3-F442A cells and MEFs is not so surprising, given that STAT5 is known to be recruited to multiple binding sites in the GH receptor (26), where it is rapidly phosphorylated by JAK2 and released from the GH receptor. It then migrates to the nucleus where it is thought to undergo dephosphorylation and then recycle back to the GH receptor for reactivation (36). The conclusion that levels of STAT5 rather than levels of JAK2 are sometimes rate-limiting for GH activation of STAT5 would be consistent with the finding of Yang et al. (6) using both 3T3-F442A cells and γ2A cells expressing ectopic GH receptor and JAK2. When these cells were treated with a dimerized form of the GH antagonist G120R, GH activation of STAT5 was maintained at normal levels even though levels of JAK2 activation are greatly suppressed. These results emphasize the need to consider the rate-limiting step in instances in which one GH signaling pathway (e.g. GH activation of STAT5) is inhibited to a lesser extent other GH signaling pathways. We also noticed that in contrast to the 3T3-F442A cells, in H4IIE cells, GH's ability to activate STAT5 appears to be more closely linked to levels of JAK2. In JAK2 shRNA cells, the reduction in

levels of GH-activated STAT5 was similar to the reduction in levels of JAK2. This finding suggests that the rate-limiting step for a particular GH signaling pathway may vary between cell types.

In conclusion, our results using pharmacological inhibitors of Src family kinases and cells with reduced levels of JAK2 using shRNA suggest that JAK2 and not a Src family kinase, is the primary kinase responsible for GH activation of STATs 1, 3, and 5, ERKs 1 and 2, and Akt in the well characterized, highly GH-responsive 3T3-F442A preadipocytes and H4IIE hepatoma cells. Studies using JAK2 and Src-deficient MEFs further support the hypothesis that GH is capable of activating STATs 3 and 5, ERKs 1 and 2, and Akt in the absence of Src family kinases and is incapable of activating STAT5 in the absence of JAK2. It is conceivable, however, that in different cell lines, perhaps where the ratio of Src family kinases to JAK2 is naturally or artificially high, Src family kinases are able to substitute for some or all of the actions of JAK2. One can also envision the levels of some as yet unidentified accessory proteins shifting the balance between JAK2 and Src in ways that we do not yet understand. Finally, our data provide a reminder that some signaling pathways are more tightly coupled to the level of activation of JAK2 than others and that this level of coupling is likely to vary between cell types. Thus, titrating the level of JAK2 activity should enable one to preferentially stimulate or inhibit some pathways more than others in different cell types.

Materials and Methods

Reagents: Recombinant 22,000-Da human GH was a kind gift from Eli Lilly & Co. (Indianapolis, IN). Dulbecco's Modified Eagle Medium (DMEM) was from Cambrex. Swims S-77 powder, L-cystine and L-glutamine were from United States Biological. Fetal bovine serum (FBS) was from Hyclone. Calf serum was from Atlanta Biologica. Sodium bicarbonate powder was from Mallinckrodt. Calcium chloride dihydrate, puromycin and polybrene (hexadimethrine bromide) were from Sigma. The antibiotic-antimycotic solution, trypsin-EDTA and Magic Mark XP western standards were from Invitrogen. Aprotinin, leupeptin, and Triton X-100 were from Roche. Recombinant protein A-agarose was from Repligen. Hybond-C Extra nitrocellulose was from Amersham Biosciences. Src family kinase inhibitors PP1 and PP2 were from BioMol (Plymouth Meeting, PA). PP3 was from Calbiochem. The mammalian expression vector prk5 encoding wild-type murine JAK2 (GI: 309463) was a generous gift from Dr. J. Ihle (St. Jude Children's Hospital, Memphis, TN) (203).

Antibodies: Antibodies recognizing a peptide containing phosphorylated tyrosines 1007 and 1008 of JAK2 (αpY1007/1008, cat. #07-606); phosphotyrosines (αPY) (4G10, cat. #05-321); and phospho STAT1 (αpY701-STAT1, cat. #06-657) were from Upstate Biotechnology, Inc. Antibody recognizing total STAT1 was from Transduction Laboratories (cat. # S21120). Antibody recognizing both total STAT5b and total STAT5a (αSTAT5, cat. # sc-1656) was from Santa Cruz Biotechnology, Inc. Antibody recognizing both phosphoSTAT5a and phospho STAT5b (αpY694-STAT5, cat. #71-6900) was from Zymed Laboratories, Inc. Antibodies recognizing phosphoSTAT3 (αpY705-STAT3, cat. #9131), total STAT3 (αSTAT3, cat #4904), phosphoERKs 1 and 2 (αpT202/pY204-ERK1/2, cat. #9106), total ERKs 1 and 2 (αERK1/2, cat. #4695), phosphoAkt (αpS473-Akt, cat. #4058),

total Akt (αAkt, cat. #9272) and phosphoSrc (αpY416-Src, cat. #2113) were from Cell Signaling Technology. Mouse monoclonal antibody recognizing Src was from Dr. Tony Hunter (Salk Institute). Mouse monoclonal antibody recognizing total JAK2 and used for immunoblotting was from BioSource International, Inc. Polyclonal antibody used for JAK2 immunoprecipitation was raised against a peptide corresponding to amino acids 758 to 776 of murine JAK2 and prepared by our laboratory in conjunction with Pel-Freez Biologicals (5). Polyclonal αGHBP antibody used for GH receptor immunoprecipitation was from Dr. William Baumbach (American Cyanamid Company, Princeton, N.J.). Polyclonal antibody (AL47) used for GH receptor immunoblot was a kind gift from Dr. Stuart Frank (University of Alabama) (204). IRDye 800 conjugated affinity purified anti-mouse IgG and anti-rabbit IgG were from Rockland.

Gene silencing by shRNA and retroviral infection: The target sequences of murine and rat JAK2 were 5'- GGAGAGTATCTGAAGTTTC-3' (205) and 5' - GGAATGGCTTGCCTTACAA-3' (206), respectively. Oligonucleotides were annealed and subcloned into pSuperior.retro.puro (Oligoengine) at BglII and XhoI sites. A control sequence of 5'- UUCUCCGAACGUGUCACGU-3' with no known target (Qiagen-Xeragon, Germantown, MD) was also cloned into the same vector. Retroviral infection was performed according to Erickson et al. (207). In brief, the recombinant plasmids were transfected into 293T cells by calcium phosphate coprecipitation together with the viral packaging vectors SV-E-MLV-env and SVψ-E-MLV (208). 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 subconfluent (~30%) 3T3-F442 cells or H4IIE cells. The infection protocol was repeated twice with intervals of 8-16 h. When cells achieved ~ 80% confluence, they were trypsinized and cells expressing JAK2

shRNA were stably selected in medium containing 2 μg/ml (3T3-F442A cells) or 40 μg/ml (H4IIE cells) puromycin.

Cell culture and transfection: The stock of murine 3T3-F442A preadipocytes was kindly provided by H. Green (Harvard University). H4IIE rat hepatoma cells were a kind gift from J. Messina (University of Alabama Birmingham School of Medicine). SYF (Src/yes/fyn) triple knock-out MEFs were kindly provide by P. Soriano (Univ. of Washington, Seattle) (192). JAK2$^{-/-}$ MEFs were a kind gift of J. Ihle (St. Jude Children's Hospital, Memphis, TN) (209). 3T3-F442A cells and 293T cells were grown in DMEM supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 8% calf serum. H4IIE cells were grown in SWIMS 77 medium supplemented with 5% FBS, 26.2 mM sodium bicarbonate, 4 mM L-glutamine, 98 μM L-cystine and 1.8 mM calcium chloride dihydrate. MEFs were grown in DMEM supplemented with 8% FBS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin per ml. MEFs were transiently transfected using lipofectamine 2000 (Invitrogen, CA). All cells were incubated overnight in serum-free medium containing 1% bovine serum albumin before treatment with 100 μM PP1, PP2, or PP3 and/or GH (500 ng/ml). All experiments were carried out at 37°C.

Immunoprecipitation and immunoblotting: For all experiments, cells were grown in 10 cm culture dishes. After GH treatment, cells were washed and solubilized in lysis buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, [pH 7.5]), containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. 1% Triton X-100 was used in place of 0.1% Triton X-100 to lyse cells for GH receptor studies. The supernatant was collected. For H4IIE cells, 50% of the supernatant was incubated with αJAK2 on ice for 2 h followed by protein G-agarose beads (GE) rotating at 4°C for 1 h. For GH receptor immunoprecipitation, 60% of the supernatant was incubated

with αGHBP on ice for 2 h followed by protein G-agarose beads (GE) rotating at 4°C for 1 h. The beads were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% ß-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue). Eluted proteins as well as proteins in cell lysates prepared in the same buffer were separated by SDS-PAGE, using 10% polyacrylamide gels and an acrylamide:bis acrylamide ratio of 30:0.5. Bands on Western blots represent 12.5% of the total lysate from a 10 cm culture plate. For immunoblotting, proteins in the gel were transferred to nitrocellulose and detected by immunoblotting with the indicated antibody using the ODYSSEY Infrared Imaging System (LI-COR Bio-sciences). The intensity of the bands in immunoblots was quantified using Li-Cor Odyssey 2.1 software. Values for phosphorylated proteins were normalized for total levels of that protein. For the shRNA experiments, JAK2 protein levels were normalized for total Src (H4IIE) or total ERK1/2 (3T3-F442A) protein levels. Every experiment was carried out at least twice with similar results. Most were performed 3 or more times (number indicated in the figure legends) with similar results.

Acknowledgements

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Chapter 3

Growth hormone induces SH2B1β and JAK2 to form a complex with the novel SH2B1β-interacting partner, βII-spectrin, and induces βII-spectrin re-localization

Abstract

SH2B1β is a multifunctional adaptor protein that modulates processes involving regulation of the cytoskeleton such as cellular motility and differentiation. This raises the possibility that SH2B1β interacts with components of the cytoskeleton or proteins that regulate the cytoskeleton. To identify novel SH2B1β interacting proteins, a yeast-two-hybrid assay was performed. The C-terminal 158 amino acids of the cytoskeleton structural protein, βIIΣ1-spectrin, were identified as interacting with the N-terminal 260 amino acids of SH2B1β. Subsequent co-immunoprecipitation assays revealed that SH2B1β amino acids 105-150 interact with βIIΣ1-spectrin amino acids 2200-2358, and confocal microscopy experiments showed that βIIΣ1-spectrin co-localizes with both SH2B1β and the SH2B1β-binding partner, JAK2, at the plasma membrane (pm)/cytoskeleton. Phosphorylation of spectrins has previously been shown to regulate spectrin localization and/or function, prompting us to assess the possibility that JAK2 phosphorylates βII-spectrin. Co-expression of JAK2 with βIIΣ1-spectrin and the βIIΣ1-spectrin splice variant, βIIΣ2-spectrin, resulted in tyrosyl phosphorylation of both βII-spectrin isoforms. Interestingly, this phosphorylation was dependent on co-expression

of SH2B1β. Finally, growth hormone (GH) stimulation induced an SH2B1β-dependent formation of a complex of endogenous βII-spectrin, SH2B1 and JAK2 in 3T3-F442A cells and a redistribution of βIIΣ2-spectrin from cell-cell contacts to the cytoplasm in H4IIE cells. These results identify a novel interaction between SH2B1β, βIIΣ1-spectrin and JAK2, and uncover an SH2B1β-dependent phosphorylation of βII-spectrin by JAK2 that may play a role in GH-mediated redistribution of βII-spectrin.

Introduction

Adaptor proteins are generally characterized as proteins that lack intrinsic enzymatic activity yet serve as important modulators of signal transduction systems by recruiting and linking together additional signaling molecules. Within the SH2B family of adaptor proteins, SH2B1 is known to modulate signaling induced by multiple hormones and growth factors (reviewed in (101)). SH2B1 mRNA is alternatively spliced into four isoforms, resulting in four proteins (α, β, γ, δ) that share an N-terminal dimerization domain (DD), nuclear localization sequence (NLS) and nuclear export sequence (NES), and a C-terminal pleckstrin homology (PH) and Src homology 2 (SH2) domain (see schematic in Fig. 1). Each isoform contains unique sequences C-terminal to the SH2 domain (105, 210).

We and others have shown previously that SH2B1 modulates hormone and growth factor induced cellular responses that require regulation and/or modification of the actin-based cytoskeleton. These responses include nerve growth factor (NGF)-induced neurite outgrowth (109, 110); GH and platelet-derived growth factor (PDGF)-induced cell ruffling and pinocytosis (132); GH-induced phagokinesis, lamellipodia activity and cell migration (112); and PDGF and insulin-like growth factor 1 (IGF-1)-induced mitogenesis (105). SH2B1β is also able to regulate actin-based bacterial motility (134). SH2B1β has been shown to localize with filamentous actin in ruffles (132), interact with the cytoskeleton remodeling proteins, Rac (112) and (bacterial) ActA (134), and cross-link actin filaments (113). Interestingly, SH2B1β C-terminal truncation and point mutants act as dominant negative proteins in many of the above processes (110, 112, 132), suggesting that the N-terminus of SH2B1β interacts with one or more proteins that are important regulators of the actin cytoskeleton.

Spectrins are large (>2000 amino acids) rod-like proteins that reside at the pm interface and act to cross-link actin filaments, transmembrane proteins and lipids either directly or indirectly through spectrin binding partners (reviewed in (131, 211). In this way, spectrins are critical organizers and mediators of cellular signaling, adhesion, mechanical stability and polarity (reviewed in (130). Cytoskeletal spectrins predominantly exist as αβ heterodimers that organize in a head-to-tail fashion to form heterotetramers (212, 213). α and β spectrins each contain PH domains as well as actin-binding calponin homology (CH) domains. α-spectrins contain an additional Src homology 3 (SH3) domain and a calmodulin-related domain. α and β spectrins also contain many (between 16 and 30) adjoining spectrin repeats, which typically consist of 106 amino acid motifs (214) that form triple helical coiled coils (215, 216). In humans, two α-spectrin genes (αI and αII) have been identified along with 6 β-spectrin genes (βI, βII, βIII, βIV, βV and β_{golgi}). βI, βII and βIV-spectrin mRNA each undergo alternative splicing to produce multiple protein isoforms (eg. βIIΣ1, βIIΣ2), potentially increasing the complexity of spectrin dimer and tetramer composition within the cytoskeleton (reviewed in (217)).

Several groups have shown that both function and localization of α and β spectrins can be regulated by phosphorylation. The earliest report (performed prior to identification of differing spectrin isoforms) found that phosphorylation of spectrin dramatically increased its ability to bind actin and promote actin polymerization (218). More recently, TGF-β has been found to induce serine phosphorylation and translocation of βIIΣ2-spectrin (also named ELF for embryonic liver fodrin) from the plasma membrane into the nucleus in HepG2 cells (219). βIIΣ2-spectrin is also thought to be serine and threonine phosphorylated by protein kinase casein kinase 2 (PKCK2) and/or protein kinase A (PKA) in response to cAMP, which may decrease its affinity for αII-spectrin and

be necessary for neuritogenesis in PC12 cells (220). In addition, serine phosphorylation is correlated with a redistribution of βII-spectrin from the membrane to the cytosol during mitosis in CHO and HeLa cells (221). Casein kinase I-mediated βI-spectrin phosphorylation has been shown to decrease mechanical stability of erythrocyte membranes (222). Finally, tyrosine phosphorylation of αII-spectrin is thought to induce calpain-mediated cleavage (223).

Here we identify βIIΣ1-spectrin as a SH2B1β binding partner and show that JAK2 is able to phosphorylate both βIIΣ1 and βIIΣ2-spectrin in an SH2B1β-dependent manner. We also show that GH stimulation induces the formation of a βII-spectrin/SH2B1/JAK2 complex in 3T3-F442A cells and causes a shift in subcellular localization of BII spectrin in H4IIE hepatoma cells, raising the possibility that some of SH2B1β's regulatory effects on GH-mediated cytoskeletal reorganization may be mediated through βII-spectrin.

Results

Amino acids 105-150 of SH2B1β interact with amino acids 2200-2358 of βIIΣ1-spectrin. SH2B1β C-terminal truncation and point mutants act as dominant negative proteins in assays involving cytoskeletal reorganization (112, 132), suggesting that the N-terminus of SH2B1β may bind unknown proteins that are functionally relevant for this process. To identify novel N-terminal SH2B1β-interacting proteins, a yeast-2-hybrid assay was utilized to screen a rat adipose cDNA library using amino acids 1-260 of SH2B1β as bait (see Fig. 3.1 for SH2B1β schematic). From 5.85 x 10^6 initial transformants, 22 LacZ+/Leu+ colonies were selected for further characterization. One of these positives comprised amino acids 2200-2358 of the cytoskeletal protein, βIIΣ1-spectrin (Fig. 3.1). To verify the yeast-two-hybrid assay interaction between the C-terminus of βIIΣ1-spectrin and the N-terminus of SH2B1β, as well as to more precisely define the SH2B1β region of interaction, co-immunoprecipitation assays were performed. Myc-tagged full-length and C-terminal SH2B1β truncation mutants were expressed with HA-tagged βIIΣ1-spectrin (2200-2358) in 293T cells (Fig. 3.2A). Myc-tagged SH2B1β (1-670), (1-260), (1-200) and (1-150) all co-immunoprecipitated with HA-βIIΣ1-spectrin (2200-2358) (Fig. 3.2A, lanes 2, 3, 4, and 5), while myc-SH2B1β (1-105) failed to co-immunoprecipitate with HA-βIIΣ1-spectrin (2200-2358) (lane 6). These results indicate that the SH2B1β-spectrin interaction is mediated through amino acids 105-150 of SH2B1β and amino acids 2200-2358 of βIIΣ1spectrin.

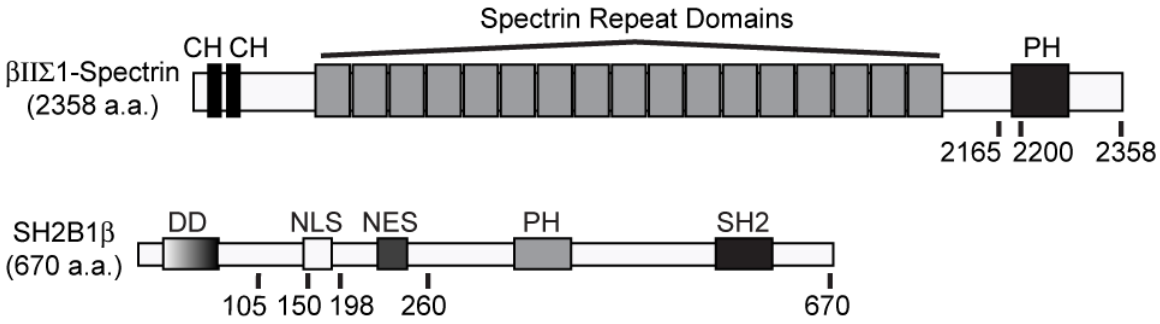

Figure 3.1. Schematic representations of βIIΣ1-spectrin and SH2B1β. Abbreviations used are DD for dimerization domain, NLS for nuclear localization sequence, NES for nuclear export sequence, PH for pleckstrin homology domain, SH2 for SH2 domain and CH for calponin homology domain. Numbers refer to amino acid positions.

We have previously characterized several SH2B1β domains as being crucial for the subcellular localization and/or function of SH2B1β, including the polybasic NLS/pm-localization region, the NES and the SH2 domain. Mutation or deletion of the NLS/pm localization region inhibits SH2B1β from both binding the pm (Maures, et al. Manuscript in preparation) and cycling through the nucleus (107) and prevents SH2B1 from enhancing neurite outgrowth (106). Mutation or deletion of the NES causes nuclear accumulation of SH2B1β and also prevents SH2B1 enhancement of NGF-induced neurite outgrowth (107). Mutation and/or deletion of the SH2 domain results in a dominant negative phenotype for GH-induced cell ruffling (132), lamellipodia activity, phagokinesis and motility (112) and NGF-induced neurite outgrowth. Each of the above processes require cytoskeletal remodeling, therefore, we sought to determine whether any of these mutations disrupt the interaction of SH2B1β with βIIΣ1-spectrin (2200-2358). Co-immunoprecipitation experiments showed that myc-tagged βIIΣ1-spectrin (2200-2358) interacts with GFP-tagged SH2B1β lacking the NLS/polybasic region

(Δ148-198, Fig. 3.2B, lane 2), lacking a functional NES (mNES, Fig. 3.2, lane 3) or lacking a functional SH2 domain (R555E, Fig. 3.2B, lane 4).

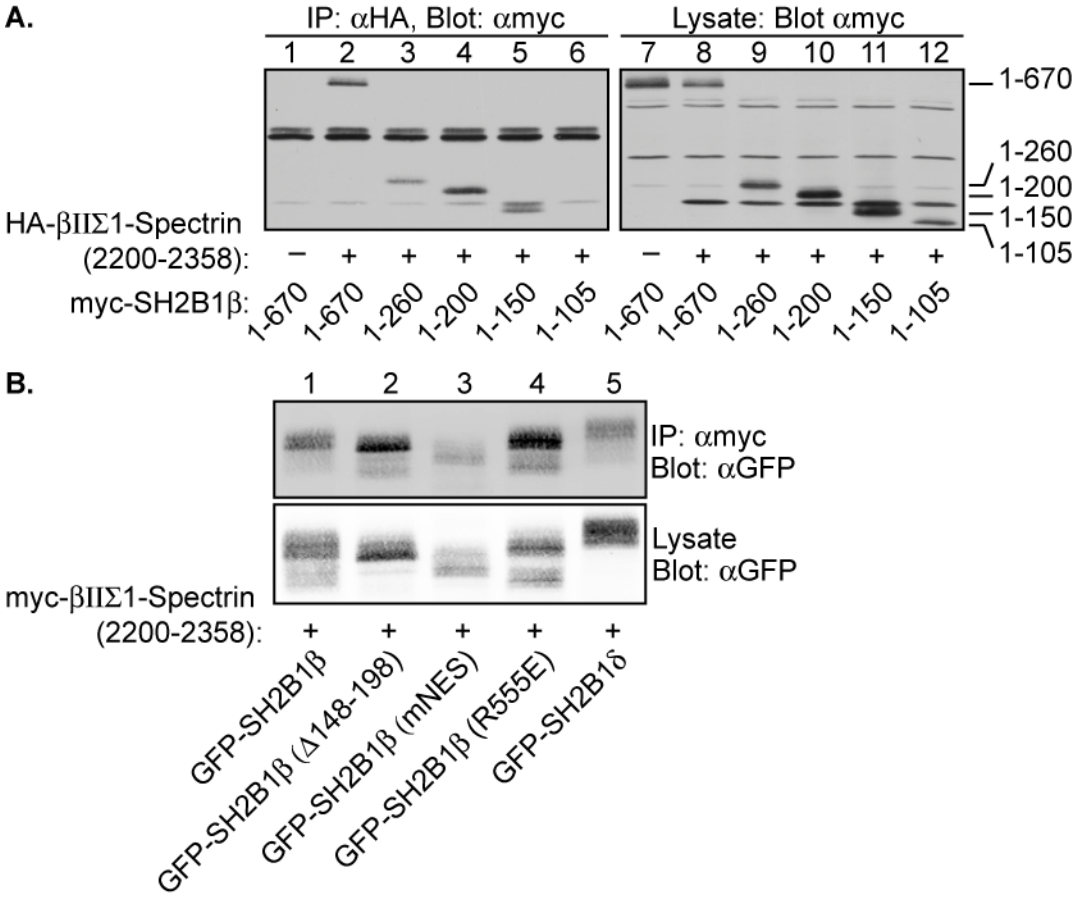

Figure 3.2. Amino acids 105-150 of SH2B1β interact with amino acids 2200-2358 of βIIΣ1-spectrin. A) HA-tagged βIIΣ1-spectin (2200-2358) was expressed with myc-tagged WT SH2B1β or SH2B1β truncation mutants in 293T cells as indicated. Proteins from cell lysates were blotted with αmyc (right panel) or immunoprecipitated with αHA and blotted with αmyc (left panel). B) myc-tagged βIIΣ1-spectrin (2200-2358) was expressed with GFP-tagged WT and mutant SH2B1β and WT SH2B1δ in 293T cells. Proteins from cell lysates were blotted with αGFP (bottom panel) or immunoprecipitated with αmyc and blotted with αGFP (top panel).

These results indicate that the NLS, NES and SH2 domains of SH2B1β do not affect the ability of with SH2B1 to interact with aa 2200-2358 of βIIΣ1-spectrin. GFP-SH2B1δ also co-immunoprecipitated with myc-βIIΣ1-spectrin (2200-2358) (Fig. 3.2B,

lane 5). This latter finding suggests that βIIΣ1-spectrin interacts with multiple SH2B1 isoforms, consistent with the region of interaction lying within the region of SH2B1 shared among all 4 SH2B1 isoforms. However, the findings that βIIΣ1-spectrin (2200-2358) interacts with SH2B1β (Δ148-198), (mNES), and SH2B1δ were somewhat unexpected, given that βIIΣ1-spectrin is characterized as a pm protein, while SH2B1δ primarily localizes to nucleoli (108). SH2B1β (Δ148-198) has been shown to be primarily cytoplasmic and mNES resides primarily in the nucleus. We therefore wondered whether βIIΣ1 spectrin 2200-2358 might be mislocalized.

SH2B1 is able to alter βIIΣ1-spectrin (2200-2358) subcellular localizaton.

Using confocal microscopy to visualize βIIΣ1-spectrin in living 293T cells, we found βIIΣ1-spectrin (2200-2358) to localize to the cytoplasm and nucleus (Fig. 3.3A-C), revealing that βIIΣ1-spectrin (2200-2358) is not properly targeted to the pm. GFP-SH2B1β localized to both the cytoplasm and pm (see Fig. 3.5B) consistent with previous reports (224), while GFP-SH2B1δ appeared to localize primarily to nucleoli (Fig. 3.3D-F) as reported previously (108). When co-expressed with GFP-SH2B1β, mCherry-βIIΣ1-spectrin (2200-2358) remained predominantly in the cytoplasm and nucleus, although some pm localization was observed (Fig. 3.3G-I). When co-expressed with GFP-SH2B1δ, mCherry-βIIΣ1-spectrin (2200-2358) was nearly completely localized to nucleoli (Fig. 3J-L). These results indicate that SH2B1 is able to alter the subcellular localization of βIIΣ1-spectrin (2200-2358), suggesting that the interaction between SH2B1 and βIIΣ1-spectrin is robust. Interestingly, although spectrins are classically described as membrane/cytoskeletal proteins, several recent reports show that some spectrins are targeted to the nucleus (reviewed in (225)) and nucleoli (226, 227) raising the possibility that a nuclear/nucleolar interaction between SH2B1 and βIIΣ1-spectrin may be functionally relevant.

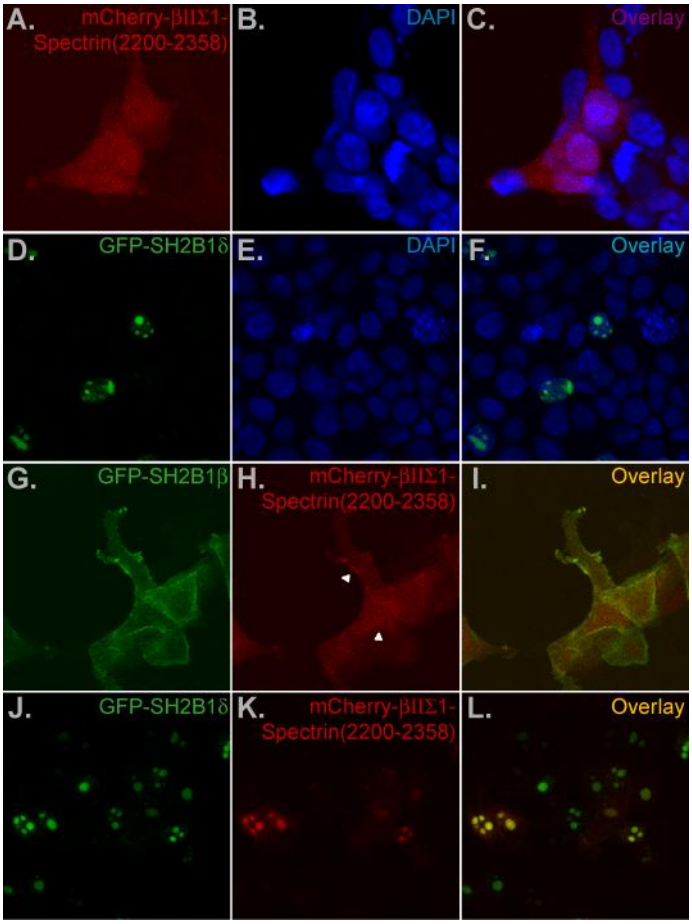

Figure 3.3. SH2B1 is able to alter the localization of βIIΣ1-spectrin (2200-2358). mCherry-βIIΣ1-spectrin (2200-2358), GFP-SH2B1β and GFP-SH2B1δ were expressed alone or in combination in 293T cells as indicated. Cells were fixed and imaged by confocal microscopy. Cells expressing mCherry-βIIΣ1-spectrin (2200-2358) or GFP-SH2B1δ alone were DAPI stained to visualize nuclei. Arrows in H indicate co-localization of mCherry-βIIΣ1-spectrin (2200-2358) and GFP-SH2B1β at the plasma membrane.

βIIΣ1-spectrin (2165-2358) recruits cytosolic SH2B1β (Δ148-198) to the pm.

βIIΣ1-spectrin is well characterized as a pm associated protein, indicating that the cytosolic/nuclear localization exhibited by βIIΣ1-spectrin (2200-2358) is atypical. Spectrin is thought to participate in the polarization of cuboidal epithelial cells (228, 229) and, therefore, cuboidal epithelial cells, such as MDCK cells, are often used to assess βII-spectrin localization and function. βIIΣ1-spectrin (2200-2358) expressed in MDCK

cells also localized to both the cytoplasm and nucleus (Fig. 3.4C), indicating that this localization pattern was not an artifact of expression in 293T cells (Fig. 3.4A). PH domains are known phospholipid and protein-protein interaction domains (reviewed in (230)), and the spectrin PH domain has been shown to bind phospholipids *in vitro* (231, 232) and be targeted to the pm of COS7 cells (233). βIIΣ1-spectrin (2200-2358) consists of the extreme C-terminus of βIIΣ-spectrin, and contains most, but not all, of the spectrin PH domain. Addition of 35 amino acids to βIIΣ1-spectrin (2200-2358) produced a βIIΣ1-spectrin C-terminal fragment that contains an intact PH domain [βIIΣ1-spectrin (2165-2358)]. Consistent with a previous report (233), and in contrast to βIIΣ1-spectrin (2200-2358) (Fig. 4A, 4C), βIIΣ1-spectrin (2165-2358) localized almost entirely to the pm in both 293T cells (Fig. 3.4B) and MDCK cells (Fig. 3.4D).

We next asked if, in addition to SH2B1 being able to alter C-terminal βIIΣ1-spectrin localization (Fig. 3.3), whether pm localized βIIΣ1-spectrin (2165-2358) could recruit SH2B1β to the pm. To this end, SH2B1β (Δ148-198), which exhibits decreased pm and increased cytosolic localization (Maures, et al. Manuscript in preparation) compared to WT SH2B1β, was expressed with or without βIIΣ1-spectrin (2165-2358) in 293T cells. Cell lysates were separated into membrane and cytosolic fractions and the relative distribution of SH2B1β (Δ148-198) was assessed (Fig. 3.4E). When expressed with βIIΣ1-spectrin (2165-2358), more SH2B1β (Δ148-198) was found in the membrane fraction than when SH2B1β (Δ148-198) was expressed alone (compare Fig. 3.4E, top panel, lanes 1 and 2). Conversely, when expressed with βIIΣ1-spectrin (2165-2358), less SH2B1β (Δ148-198) was found in the cytosolic fraction than when SH2B1β (Δ148-198) was expressed alone (compare Fig. 3.4E, top panel, lanes 3 and 4). Because β-spectrins are able to organize structural and signaling proteins to specific regions within

the pm (reviewed in (234)), these results suggest that βIIΣ1-spectrin may be involved in the targeting of SH2B1β to distinct regions within the pm.

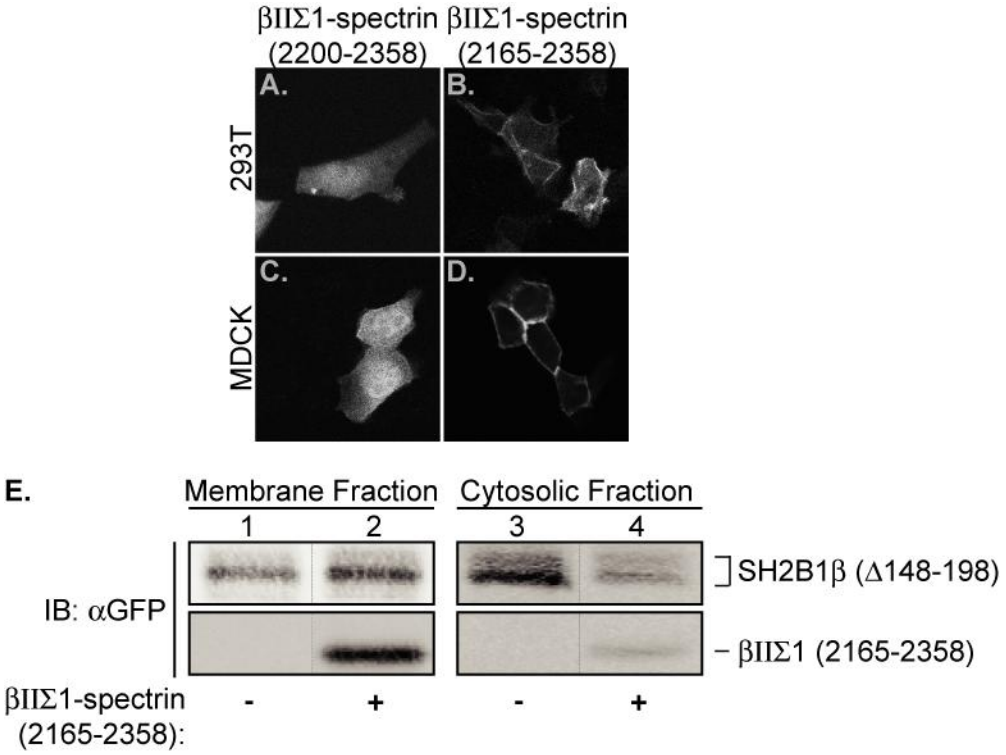

Figure 3.4. βIIΣ1-spectrin (2165-2358) is targeted to the pm and is able to alter SH2B1β (Δ148-198) localization. GFP-βIIΣ1-spectrin (2200-2358) was expressed in 293T (A) or MDCK (C) cells. GFP-βIIΣ1-spectrin (2165-2358) was expressed in 293T (B) or MDCK (D) cells. Cells were fixed and imaged by confocal microscopy. E) GFP-SH2B1β (Δ148-198) was expressed alone or with βIIΣ1-spectrin (2165-2358) in 293T cells. Cells were lysed, separated into membrane and cytosolic fractions and blotted with αGFP.

βIIΣ1-spectrin co-localizes with both SH2B1β and JAK2. To determine if full-length βIIΣ1-spectrin co-localizes with full-length SH2B1β, βIIΣ1-spectrin and SH2B1β were expressed alone or together in 293T cells (Fig. 3.5). As expected, when expressed alone GFP-βIIΣ1-spectrin (Fig. 3.5A) and GFP-SH2B1β (Fig. 3.5B) each localized

primarily to the pm. To begin to explore a potential functional significance of the βIIΣ1-spectrin/SH2B1β interaction, we investigated the possibility that the SH2B1β binding partner, JAK2 ((100), reviewed in (101)), is also a component of this interaction. Consistent with previous reports demonstrating JAK2 localization at the pm in HEK293T and γ2A cells (235, 236), GFP-JAK2 localized primarily to the pm in 293T cells (Fig. 5C). CFP-JAK2, GFP-βIIΣ1-spectrin and mCherry-SH2B1β all co-localized at the pm when all three proteins were expressed in 293T cells (Fig. 3.5D-G). These results raise the possibility that βIIΣ1-spectrin exists in a complex with SH2B1β and JAK2 at the pm and that βIIΣ1-spectrin is a novel target of JAK2 kinase activity.

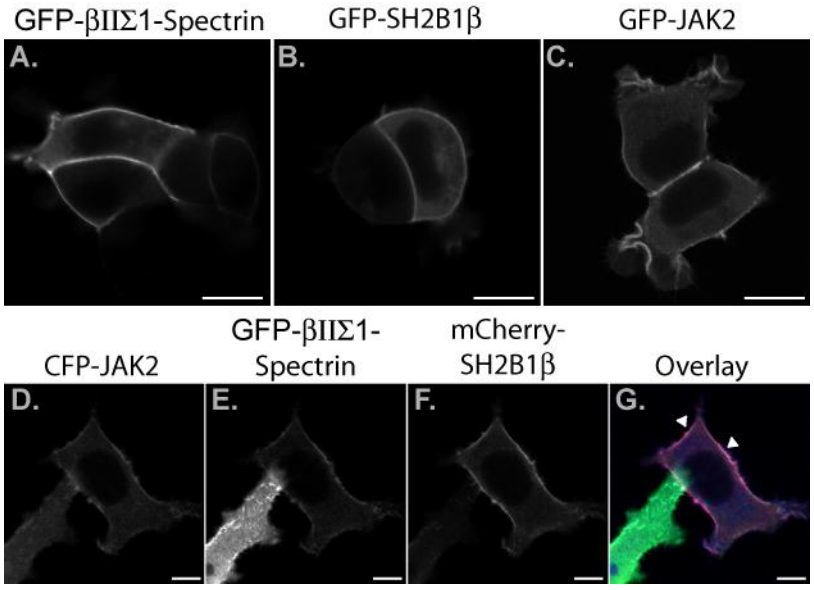

Figure 3.5. βIIΣ1-spectrin co-localizes with SH2B1β and JAK2 at the plasma membrane. Fluorescently tagged βIIΣ1-spectrin, SH2B1β and JAK2 were expressed alone (Panels A-C) or together (Panels D-G) in 293T cells and image by live confocal microscopy. Arrows in Panel G indicate co-localization on the pm. Scale bars = 10 μm.

SH2B1β is necessary JAK2-induced tyrosyl phosphorylation of βIIΣ1-spectrin and βIIΣ2-spectrin. αII-spectrin is known to be tyrosyl phosphorylated by Src kinase in COS cells (237), and, although β-spectrin can be tyrosyl phosphorylated by insulin receptor kinase (238, 239), Src kinase and epidermal growth factor receptor kinase (239) *in vitro*, no studies have yet shown β-spectrins to be tyrosyl phosphorylated *in vivo*. To determine if JAK2 is able to induce tyrosyl phosphorylation of βIIΣ1-spectrin, GFP-βIIΣ1-spectrin was expressed alone (Fig. 3.6A, lane 3) or with JAK2 (Fig. 3.6A, lane 2) in 293T cells. Co-expression of βIIΣ1-spectrin and JAK2 did not result in βIIΣ1-spectrin tyrosyl phosphorylation (compare Fig. 3.6A, top panel, lanes 2 and 3). However, when Flag-SH2B1β was co-expressed with GFP-βIIΣ1-spectrin and JAK2, GFP-βIIΣ1-spectrin was tyrosyl phosphorylated (Fig. 3.6A, top panel, lane 4). The alternative splice variant of βIIΣ1-spectrin, βIIΣ2-spectrin, is ~92% identical to βIIΣ1-spectrin at the amino acid level, differing in sequence at the N and C-termini. To determine if JAK2 is also able to mediate tyrosyl phosphorylation of βIIΣ2-spectrin, myc-βIIΣ2-spectrin and JAK2 were expressed without (Fig. 3.6B, lane 1) or with (Fig. 3.6B, lane 2) myc-SH2B1β. As with βIIΣ1-spectrin, JAK2 was only able to induce tyrosyl phosphorylation of βIIΣ2-spectrin when SH2B1β was co-expressed.

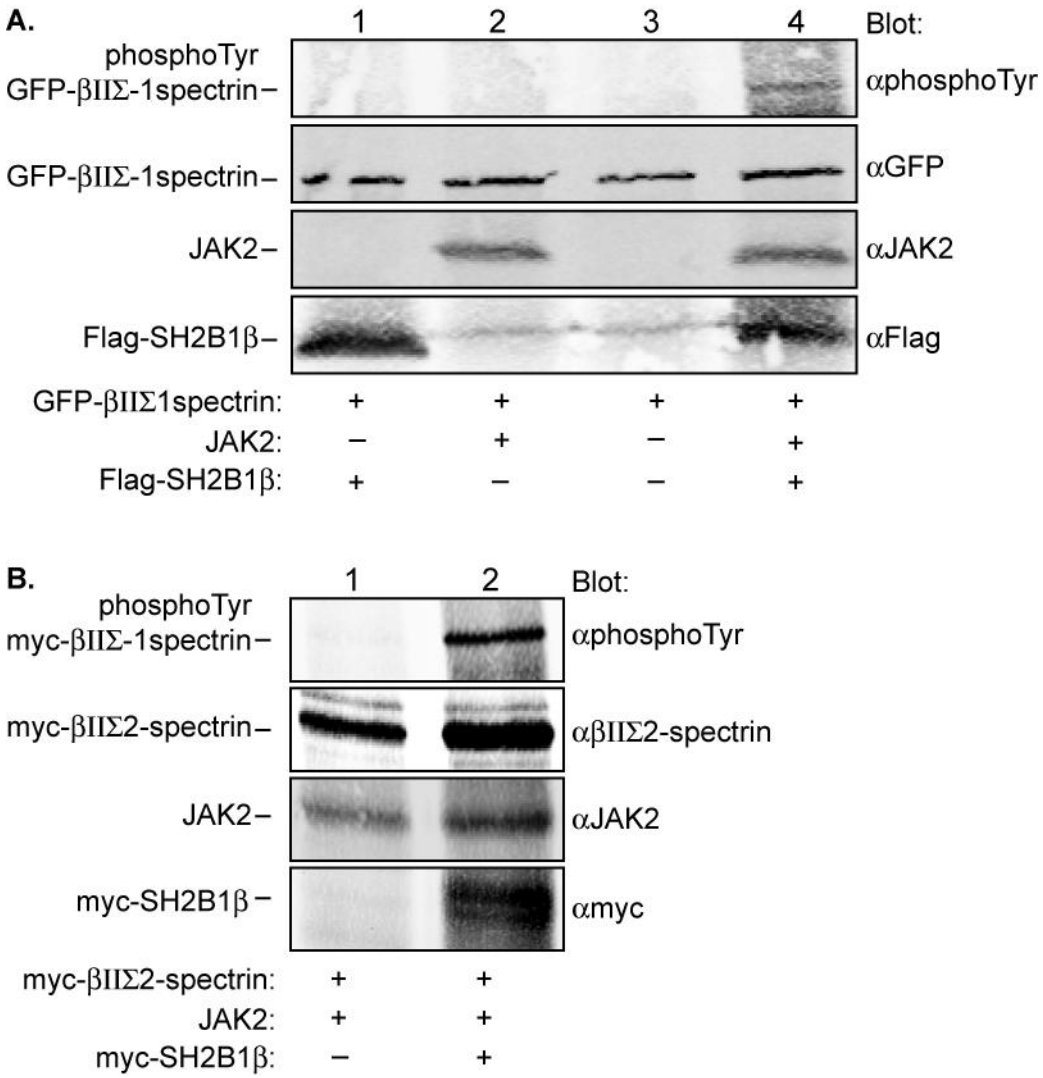

Figure 3.6. JAK2 induces SH2B1β-dependent tyrosyl phosphorylation of βIIΣ1-spectrin and βIIΣ2-spectrin. A) GFP-βIIΣ1-spectrin, JAK2, and Flag-SH2B1β were expressed alone or in combination in 293T cells. Cell lysates were blotted with αphospho-tyrosine, αGFP, αJAK2 and αFlag as indicated. B) myc-βIIΣ2-spectrin and JAK2 were expressed with or without myc-SH2B1β in 293T cells. Cell lysates were blotted with αphospho-tyrosine, αβIIΣ2-spectrin, αJAK2 and αmyc as indicated.

GH induces an SH2B1-dependant βII-spectrin/SH2B1/JAK2 complex. 3T3-F442A cells express SH2B1 (100) and GH stimulation of these cells results in JAK2 activation (5, 240), making these cells a suitable model to test potential JAK2-mediated phosphorylation of βII-spectrin in response to ligand activation using endogenous

proteins. To determine if 3T3-F442A cells express βII-spectrin, 3T3-F442A cells were separated into a cytoskeletal-enriched fraction and a soluble fraction (see materials and methods) and blotted with αβII-spectrin (an antibody that recognizes both βIIΣ1-spectrin and βIIΣ2-spectrin) and αvinculin (Fig. 3.7A). βII-spectrin was found exclusively in the cytoskeletal fraction while vinculin was found in both the soluble and cytoskeletal fractions, consistent with previous reports (241).

We have established 3T3-F442A cells that stably express either control shRNA (shControl) or shRNA against SH2B1β (shSH2B1β) that results in a significant reduction of SH2B1β (Fig. 3.7B, bottom panel, compare lanes 1 and 3). These cells allowed us to examine the necessity of SH2B1β for GH-induced JAK2-mediated phosphorylation of βII-spectrin. 3T3-F442A shControl cells (Fig. 3.7B, lanes 1 and 2) or shSH2B1β cells (Fig. 3.7B, lanes 3 and 4) were stimulated with or without GH for 20 min, and proteins from cell lysates were immunoprecipitated with αβII-spectrin. Western blot analysis showed that although βII-spectrin was immunoprecipitated (Fig. 3.7B, panel 2), tyrosyl phosphorylated βII-spectrin was not detected in either shControl or shSH2B1β cells (Fig. 3.7B, panel 1). However, GH stimulation induced a significant increase in the association between SH2B1 and βII-spectrin in shControl cells (Fig. 3.7B, panel 8, compare lanes 1 and 2). In addition, GH stimulation induced co-immunoprecipitation of JAK2 with βII-spectrin in shControl cells (Fig. 3.7B, panel 4 compare lanes 1 and 2). As expected, a lower amount of SH2B1 co-immunoprecipitated with βII-spectrin in shSH2B1β cells due to reduced SH2B1 protein expression (Fig. 3.7B, panel 8 compare, lanes 3 and 4). Interestingly, the amount of JAK2 co-immunoprecipitating with βII-spectrin in response to GH was also reduced in shSH2B1 cells (Fig. 3.7B, panel 4, compare lanes 3 and 4), compared to shControl cells (Fig. 3.7B, panel 4, compare lanes 2 and 4). This was not due to decreased activation of JAK2, as GH induced similar

levels of tyrosyl-phosphorylated JAK2 in shControl and shSH2B1β cells (Fig. 3.7B, panel 7, compare lanes 2 and 4). These results show that βII-spectrin and SH2B1β interact in the basal state and that GH stimulation results in a significant increase in the βII-spectrin/SH2B1 interaction. In addition, these results indicate that GH stimulation induces the formation of an βII-spectrin/SH2B1β/JAK2 complex, and suggest that the presence of JAK2 in this complex is dependant on SH2B1β.

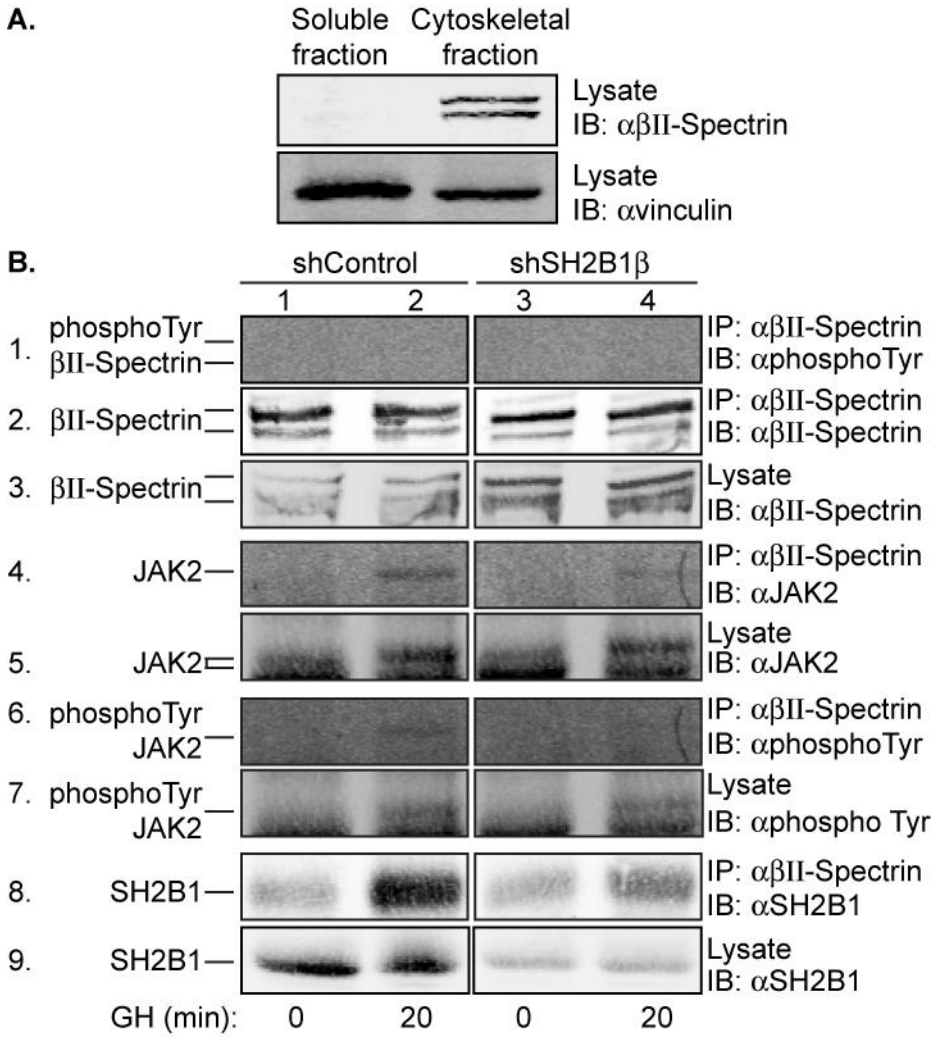

Figure 3.7. GH induces the formation of an βIIΣ1-spectrin/SH2B1/JAK2 complex. A) 3T3-F442A lysates were separated into a cytoskeletal enriched fraction and a soluble fraction and blotted with αβII-spectrin and αvinculin antibodies. B) 3T3-F442A cells were serum-deprived overnight and stimulated with or without GH for 20 min. Proteins from cell lysates were immunoprecipitated with αβII-spectrin and lysates and immunoprecipitates were blotted with αphospho-tyrosine, αβII-spectrin, αJAK2, and αSH2B1 as indicated.

GH induces subcellular redistribution of βII-spectrin. Previous reports described above suggest that phosphorylation of spectrins can alter their subcellular distribution. In HepG2 cells, TGFβ-induced phosphorylation of βIIΣ2-spectrin is reported to cause βIIΣ2-spectrin to leave the pm and translocate to the nucleus (219). To

determine if GH causes βIIΣ2 to translocate to the nucleus, H4IIE hepatoma cells were stimulated with GH or TGFβ over 30 min. Western blot analysis of βII-spectrin and proteins that are phosphorylated downstream of TGFβ (Smad3) and GH (JAK2) indicated that H4IIE cells express βII-spectrin and are responsive to both TGFβ and GH (Fig. 3.8A) (177). Immunofluorescence staining using an αβIIΣ2-spectrin antibody revealed that GH, like TGFβ, stimulates a redistribution of βIIΣ1-spectrin from cell-cell contacts to a more cytoplasmic localization within 15-30 min (Fig. 3.8B).

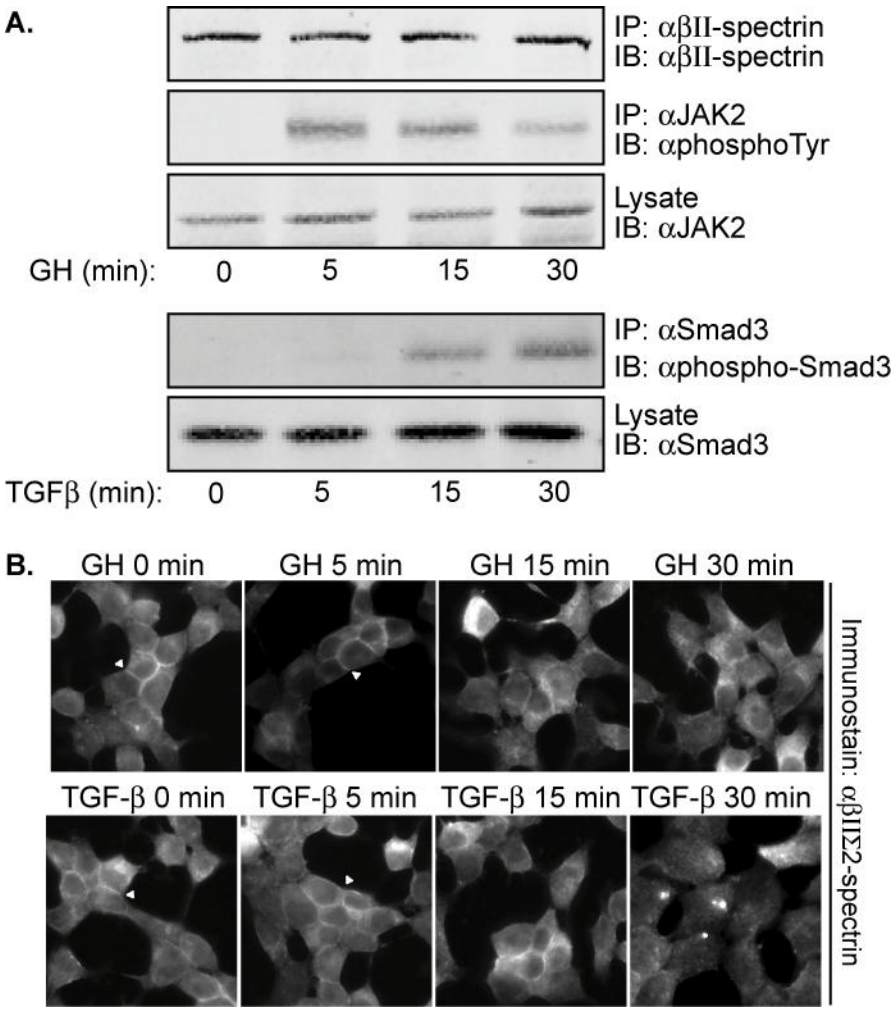

Figure 3.8. GH induces a shift in βIIΣ2-spectrin subcellular localization. H4IIE hepatoma cells were serum-deprived overnight and stimulated with or without GH (500 ng/ml) or TGF-β (100 ng/ml). A) Cells were lysed at the indicated times, and proteins from cell lysates were immunoprecipitated with αβII-spectrin or αJAK2 and lysates and immunoprecipitates were immunoblotted with αβII-spectrin, αphospho-tyrosine or αJAK2 as indicated (top panels). Alternately, proteins from cell lysates were immunoprecipitated with αSmad3 and immunoprecipitates and lysates were immunoblotted with αphospho-Smad3 or αSmad3 as indicated (bottom panels). B) Cells were fixed at the indicated times, immunostained for βIIΣ2-spectrin and visualized by widefield fluorescence microscopy. Arrows indicate βIIΣ1-spectrin localization at cell-cell contacts.

Discussion

Here we have identified βIIΣ1-spectrin as a novel SH2B1β binding partner. The region of interaction lies within amino acids 105-150 of SH2B1β and 2200-2358 of βIIΣ1-spectrin. The interaction between these proteins appears to be robust, as we have shown that SH2B1 isoforms that localize to the pm (SH2B1β) recruit cytoplasmic and nuclear-localized βIIΣ1-spectrin (2200-2358) to the pm (Fig. 3.3). Conversely, we showed that pm localized βIIΣ1-spectrin (2165-2358) recruits cytoplasmic localized SH2B1β (Δ148-198) to the pm (Fig. 3.4). These results suggest that SH2B1β and βIIΣ1-spectrin interact in the appropriate subcellular locations, and raise the possibility that the localization of each protein may be influenced by the localization of the other. In addition, it is possible that βIIΣ1-spectrin recruits SH2B1β to functionally relevant domains within the pm as spectrins have been reported to do for other proteins (reviewed in (234)). The region of interaction on SH2B1β is shared between all 4 SH2B1 isoforms, indicating that βIIΣ1-spectrin is likely to interact with all 4 SH2B1 isoforms. Indeed, we found that SH2B1δ, like SH2B1β, co-immunoprecipitates and co-localizes with βIIΣ1-spectrin (2200-2358) (Fig. 3.2). The co-localization of SH2B1δ and βIIΣ1-spectrin (2200-2358) in nucleoli was particularly interesting as several groups have observed spectrin in the nucleus and nucleoli (226, 227).

We and/or others have previously shown that βII-spectrin, SH2B1β and JAK2 all localize to the pm. Therefore, it was not surprising that we observed these three molecules to co-localize at the pm in 293T cells (Fig. 3.5). However, we present the novel finding that JAK2 induces tyrosine phosphorylation of βIIΣ1-spectrin and βIIΣ2-spectrin, and that this phosphorylation is dependent on SH2B1β (Fig. 3.6). These data raise several questions, including whether JAK2 directly phosphorylates βII-spectrins,

whether JAK2 can induce phosphorylation of βII-spectrins when co-expressed with SH2B1β lacking the spectrin-interaction region, and whether SH2B1β physically induces a JAK2-βII-spectrin interaction or, alternatively, enhances JAK2 activation to a level that is necessary for βII-spectrin phosphorylation. Some of these questions can be answered by our finding of a GH-induced formation of an endogenous βII-spectrin/SH2B1β/JAK2 complex (Fig. 3.7). We show that co-immunoprecipitation of JAK2 with βII-spectrin is dependent on the presence of SH2B1β, and that GH induces JAK2 activation to similar levels in both shControl and shSH2B1β cells. These results suggest that SH2B1β physically brings βII-spectrin and JAK2 together rather than enhancing JAK2 to a level needed to phosphorylate βII-spectrin. However, we were unable to observe GH-induced βII-spectrin tyrosyl phosphorylation in this experiment, suggesting that although GH induces the formation of a βII-spectrin/SH2B1β/JAK2 complex in 3T3-F442A cells, JAK2 may not phosphorylate βII-spectrin under these conditions. Alternatively, it is possible that under these conditions, not enough βII-spectrin was tyrosyl-phosphorylated to be observed by immunoblotting with αphospho-tyrosine or that GH induces tyrosyl-phosphorylation of βII-spectrin at a time point other than 20 min. Future experiments similar to those performed for Figure 3.7 should clarify these remaining questions.

Historically, spectrins have been known as important structural molecules for the cytoskeleton. Regulated localization of spectrins into or out of the cytoskeleton has been thought to be an important event in cytoskeletal reorganization (220, 221, 242). Spectrins have also been implicated as central nodes that serve to recruit multiple signaling molecules into specialized membrane regions (234). The SH2B1β-βIIΣ1-spectrin interaction may fit both models. βII-spectrin is able to bind both JAK2 and

SH2B1β, and therefore may serve as a node to maintain two signaling molecules in close proximity that are important for several ligands, perhaps facilitating more efficient signaling. On the other hand, data demonstrating JAK2-mediated phosphorylation of βII-spectrin and GH-induced redistribution of βIIΣ2-spectrin out of cell-cell contacts and into the cytoplasm support spectrin being a regulated cytoskeletal structural protein. In addition, SH2B1β mutants that are unable to bind the plasma membrane act as dominant negatives in the neuronal differentiation of PC12 cells, a process that is also regulated by phosphorylation of βIIΣ2-spectrin (220).

In summary, we have demonstrated that βIIΣ1-spectrin is a novel SH2B1β binding protein and that SH2B1β is required for JAK2-induced tyrosyl phosphorylation of both βIIΣ1-spectrin and βIIΣ2-spectrin. In addition, we have shown that in 3T3-F442A cells, GH stimulation induces the formation of an endogenous βII-spectrin/SH2B1/JAK2 complex. Finally, we have shown that GH induces βIIΣ2-spectrin redistribution from cell-cell contacts into the cytoplasm. Taken together, we propose a model wherein SH2B1β binds βII-spectrin at the plasma membrane, and that upon GH stimulation, active JAK2 recruits the βII-spectrin/SH2B1β complex via the SH2 domain of SH2B1β, forming a tertiary complex. Active JAK2 then phosphorylates βII-spectrin, resulting in the translocation of βII-spectrin out of the pm allowing for reorganization of the cytoskeleton. While the functional relevance of this model is beyond the scope of this study, the previously demonstrated importance of βII-spectrin, SH2B1β and GH in processes that require restructuring of the pm provide grounds for further investigation into this novel relationship. Future studies assessing the impact of the βIIΣ1-spectrin/SH2B1β/JAK2 interaction in cell motility, ruffling and neuronal differentiation assays will provide further clarification of the functional importance of this interaction.

Materials and Methods

Antibodies: The following antibodies were used for Western blotting at a dilution of 1:1000: Anti-myc mouse monoclonal antibody (αmyc) (Santa Cruz Biotechnology, Inc.), anti-HA mouse monoclonal antibody (αHA) (Covance), anti-JAK2 (αJAK2) and anti-phosphotyrosine (αPY) (4G10) mouse monoclonal antibodies (Millipore), anti-Flag M2 mouse monoclonal antibody (αFlag) (Sigma), anti-βII-spectrin rabbit polyclonal antibody (αβII spectrin) (Bethyl Laboratories, Inc.), anti-Smad3 (αSmad3) and anti-phospho-Smad3 (Ser423/425) (αpSmad3) rabbit polyclonal antibodies (Cell Signaling Technology), anti-SH2B1 [kind gift of Dr. Liangyou Rui (University of Michigan) (243)] (αSH2B1) and anti-βIIΣ2-spectrin {(αELF2), kind gift of Dr. Lopa Mishra (Georgetown University)}. For immunoprecipitation, αmyc and αHA were used at a dilution of 1:100, and αβII-spectrin was used at a dilution of 1:75. IRDye 800- and IRDye 700-conjugated affinity-purified anti-mouse IgG and anti-rabbit IgG (Rockland Immunochemicals Inc.) were used at a dilution of 1:20,000. Anti-GFP IRDye 800-conjugated goat polyclonal antibody (Rockland) (αGFP) was used at a dilution of 1:5000 for Western blotting. Anti-GFP mouse monoclonal antibody (αGFP mab) for immunoprecipitation (1:100 dilution) was from Clontech (Mountain View, CA). α-βIIΣ2-spectrin was used at a dilution of 1:100 for immunofluorescence. Alexa Fluor 594 secondary antibodies (dilution 1:1000) used for immunofluorescence were from Invitrogen.

Reagents: Recombinant 22,000-Da human GH was a kind gift from Eli Lilly & Co. Dulbecco's Modified Eagle Medium (DMEM) was from Cambrex. Fetal bovine serum (FBS) was from Hyclone. Calf serum was from Atlanta Biologicals. The antibiotic-antimycotic solution, trypsin-EDTA and Magic Mark XP western standards

were from Invitrogen. Aprotinin, leupeptin, and Triton X-100 were from Roche. Recombinant protein A-agarose was from Repligen. Hybond-C Extra nitrocellulose was from Amersham Biosciences. Paraformaldehyde was from Electron Microscopy Sciences.

Cell Culture, Transfection and Stimulation: The stock of murine 3T3-F442A fibroblasts was kindly provided by Dr. H. Green (Harvard University), 293T cells were from ATCC, MDCK cells were kindly provided by Dr. B. Margolis (University of Michigan, Ann Arbor) and H4IIE hepatoma cells were kindly provided by Dr. J. Messina (University of Alabama, Birmingham). 3T3-F442A and 293T cells were grown in DMEM supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 8% calf serum. MDCK cells were grown in Messina (University of Alabama, Birmingham). 3T3-F442A and 293T cells were grown in DMEM supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 8% fetal bovine serum. H4IIE cells were grown in SWIMS 77 medium supplemented with 5% FBS, 26.2 mM sodium bicarbonate, 4 mM L-glutamine, 98 μM L-cystine and 1.8 mM calcium chloride dihydrate. 293T cells were transiently transfected by calcium phosphate precipitation. 3T3-F442A and H4IIE cells were incubated overnight in serum-free medium before treatment with GH.

Plasmids: GFP-tagged SH2B1β, SH2B1β (R555E), SH2B1β (Δ148-198) and SH2B1β mNES were cloned as described previously (106, 107, 110). GFP-βIIΣ1-spectrin was a kind gift from Dr. Vann Bennett (Duke University) and myc-βIIΣ1-spectrin was a kind gift from Dr. Lopa Mishra (Georgetown University).

Immunofluorescence: For fixed-cell imaging, cells were fixed in 4% parformaldehyde in PBS, gently washed 3 times in PBS, permeabilized in 0.1% TritonX-

100 in phosphate buffered saline (PBS) and blocked for 30 min in PBS containing 5% normal serum from the species used for secondary antibody production. Cells were then incubated with primary antibody diluted in blocking solution for one hour. Cells were gently washed 3 times in PBS, and then incubated for 1 h with secondary antibody diluted in PBS. Cells were gently washed 3 times in ddH_2O, and then mounted on Fisherfinest Premium Microscope Slides (Fisher Scientific) using Prolong Gold Antifade mounting reagent (Invitrogen). For live cell imaging, cells were grown on no. 1.5 glass bottom dishes (MatTek Corp. Ashland, MA) and imaged in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ $6H_2O$, 2 mM NaH_2PO_4 H_2O, 10 mM HEPES, 10 mM glucose). Confocal laser scanning microscopy images were obtained with an Olympus FluoView 500 Laser Scanning Confocal Microscope. Widefield fluorescence microscopy images were obtained using a Nikon Eclipse TE200 inverted fluorescence microscope.

Immunoprecipitation and Immunoblotting: Cells were grown in 10 cm culture dishes, washed and solubilized in lysis buffer (50 mM Tris [pH 7.5], 1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, [pH 7.5]), containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. For immunoprecipitation, 60% of the supernatant was incubated with antibody on ice for 2 h followed by incubation with protein A-agarose beads rotating at 4°C for 1 h. The beads were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% ß-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue). Eluted proteins as well as proteins in cell lysates prepared in the same buffer were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 10% polyacrylamide gels and an acrylamide:bis acrylamide ratio of 30:0.5. For

immunoblotting, proteins in the gel were transferred to nitrocellulose and detected by immunoblotting with the indicated antibody.

Cell Fractionation: Membrane and cytosolic fractionation: Cells from a 10 cm dish were collected in 1.0 ml ice-cold phosphate-buffered saline containing 2 mM $Na_3(VO)_4$ (PBSV) and pelletted by centrifugation at 1000 RPM for 2 min. PBSV was aspirated, and the pellet was resuspended in 500 μl extraction buffer, (50 mM Tris-HCl pH 7.5, 50 mM beta-mercaptoethanol, 2 mM EGTA, 0.1 mM EDTA, 0.1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Samples were incubated on ice for 10 min with occasional 10 s vortexing. Samples were then pelleted by centrifugation at 1000 RPM for 1 min. The supernatant was collected and the pellet was discarded. The supernatant was centrifuged at 120,000 x g for 2 h. The supernatant was designated the cytosolic fraction and was transferred to a new tube. The pellet was designated the membrane fraction and was washed once in extraction buffer and then resuspended in 100 μl lysis buffer (50 mM Tris pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin).

Cytoskeletal enrichment fractionation (241): Cells were washed with cell solubilization buffer (10 mM PIPES, 50 mM KCl, 20 mM EGTA, 3 mM $MgCl_2$, 2 M glycerol, 2 mM NaF, 2 mM $Na_3(VO)_4$, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), then incubated on ice in CSB containing 1% TritonX-100 for exactly 5 min. The buffer containing solubilized proteins was removed and designated the soluble fraction. Plates were then washed with CSB and the remaining cytoskeletal fraction was collected with extraction buffer (20 mM Tris-HCl, 300 mM NaCl, 30 mM $MgCl_2$, 1 mM DTT, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and passed through a 28-gauge syringe 10 times. Proteins in each fraction were quantified

using the bicinchoninic acid protein assay (Pierce Biotechnology), and 50 μg of protein was used for Western blot analysis.

Acknowledgements

We thank Dr. Liangyou Rui for his gift of SH2B1 antibody, Dr. Lopa Mishra for her gifts of full-length βIIΣ2-spectrin cDNA and antibody, and Dr. Vann Bennett for his gift of full-length βIIΣ1-spectrin cDNA.

This Chapter represents a "manuscript in progress" and will be submitted for publication under the title "Growth hormone induces SH2B1β and JAK2 to form a complex with the novel SH2B1β-interacting partner, βII-spectrin, and induces βII-spectrin re-localization" by Nathan J. Lanning, Hui Jin, Steven Archer and Christin Carter-Su. Steven Archer and I collaborated on Fig. 3.2 and I provided Figs. 3.1 and 3.3-3.8.

Chapter 4

Identification and characterization of SH2B1β as a novel focal adhesion protein

Abstract

The multifunctional adaptor protein, SH2B1β, regulates diverse cellular functions, including cell motility. To gain further insight into the ability of SH2B1β to regulate the cytoskeleton and cell motility, we assessed the subcellular localization of GFP-SH2B1β in fibroblast and epithelial cells. We found GFP-SH2B1β to localize to focal adhesions via its SH2 domain. Focal adhesions are cell adhesion sites, critical nodes of cell-extracellular matrix communication and important facilitators of cell motility. Multiple protein kinase C (PKC) isoforms are found in focal adhesions and PKC activation induces the formation of focal adhesions and promotes cell motility. We provide evidence that SH2B1β is phosphorylated in response to phorbol 12-myristate 13-acetate (PMA)-induced PKC activation and show that PMA stimulation induces a rapid redistribution of SH2B1β out of focal adhesions. Multiple serines in SH2B1β lie within PKC consensus substrate phosphorylation motifs. Mutation to alanine of two of these serines (Ser 161 and 165) abrogates the ability of PMA stimulation to induce this redistribution. These mutations also significantly decrease the dynamic cycling of SH2B1β into and out of focal adhesions as analyzed by fluorescence recovery after photobleaching (FRAP) analysis. Mutation of Ser 165 to glutamate (mimicking phosphorylation) reduces the amount of SH2B1β at focal adhesions, increases the rate

of SH2B1β turnover at focal adhesions and significantly increases the number of focal adhesions per cell. Taken together, these results show that SH2B1β localizes to focal adhesions through its SH2 domain. They also provide evidence that PKC activation regulates SH2B1β focal adhesion localization through phosphorylation of SH2B1β serines 161 and/or 165 and that PKC-mediated phosphorylation of SH2B1β at serine165 regulates focal adhesion formation.

Introduction

SH2B1 is a member of the SH2B family of adaptor proteins that includes SH2B1 (formerly SH2-B/PSM), SH2B2 (formerly APS) and SH2B3 (formerly Lnk). SH2B1 mRNA is alternatively spliced to produce SH2B1α, β, γ and δ isoforms (105, 210). The translated products share a dimerization domain (DD), a nuclear localization signal (NLS) a nuclear export signal (NES), a pleckstrin homology (PH) domain and a Src homology 2 (SH2) domain (see schematic in Fig. 1), but differ at the extreme C-terminus of each protein. SH2B1β is recruited, via its SH2 domain, to multiple activated receptor tyrosine kinases and receptor-associated tyrosine kinases, enabling it to serve as an adaptor/scaffolding protein for multiple hormones and growth factors, including growth hormone (GH) (100), leptin (111), nerve growth factor (100, 109), brain derived neurotrophic factor (109), glial derived neurotrophic factor (244), insulin (210, 245), insulin-like growth factor I (246), fibroblast growth factor (247), platelet-derived growth factor (248) and hepatocyte growth factor (249). Within the context of these signaling systems, SH2B1β has been shown to enhance kinase activity, regulate gene transcription and/or modulate cytoskeletal dynamics (reviewed in (101)). These cellular effects contribute to the ability of SH2B1β to promote neuronal differentiation and maintenance of a differentiated phenotype (109, 110), regulate energy and glucose homeostasis (111), and promote cell motility (112, 113). All of these effects require either regulated intracellular transport or alterations in cell morphology, processes that rely on regulation of the actin-based cytoskeleton (reviewed in (121, 122)). SH2B1β localizes to the plasma membrane/cytoskeleton (224) and membrane ruffles (132), suggesting that SH2B1β localization to cytoskeletal structures may be important for its function. SH2B1β has been shown to regulate cytoskeletal dynamics by enhancing GH,

PDGF and prolactin-induced cell ruffling and lamellipodia formation (113, 132), and by enhancing GH-dependent cell motility (112). In addition, SH2B1β has been shown to bind activated Rac (112), cross-link actin filaments *in vitro* (113), and interact with the *Listeria monocytogenes* actin nucleating protein, ActA, to enhance bacterial actin-based motility (134). Other SH2B family members have also been implicated in regulation of the cytoskeleton. SH2B2 co-localizes with actin in B cells (135), is thought to modulate actin dynamics in mast cells (136), and interacts with the cytoskeleton regulatory proteins, Vav3 (137) and Enigma (138). SH2B3 is a binding partner of the actin binding and focal adhesion protein, filamin, although this interaction was not shown to be present in focal adhesions (139).

Focal adhesions are large integrin-based macromolecular complexes that mediate cell-extracellular matrix (ECM) attachment, facilitate direct signaling between the extracellular matrix and the cell, and facilitate cell anchorage and motility (reviewed in (128)). The number of proteins identified as localizing to focal adhesions is vast, and the number and regulation of interactions between focal adhesion proteins make these structures among the most dynamic and complex structures within a cell (see (128)for review}). Individual proteins within focal adhesions contribute to focal adhesion function through a number of mechanisms. Many proteins, such as vinculin, provide structural support for focal adhesion complexes and are generally associated with reduced cellular motility (250). Other scaffolding/adaptor proteins, such as paxillin, are generally associated with promoting migration by recruiting and assembling critical focal adhesion signaling components (251). All focal adhesion proteins are dynamically regulated so that they continually cycle in and out of focal adhesions (focal adhesion protein turnover). Modulation of this focal adhesion protein turnover can directly affect the strength and turnover of focal adhesions themselves (252), and thus cell motility. Phosphorylation of focal adhesion proteins is a major mechanism by which turnover is

regulated. Several kinases are localized to focal adhesions [e.g. focal adhesion kinase (FAK), Src family kinases, ERKs and protein kinase C (PKC)]; when activated, they phosphorylate nearby focal adhesion proteins, serving to regulate focal adhesion stability, turnover, and cell motility (129, 253). Several PKC isoforms exist, some of which (α, δ, ε) have been identified as focal adhesion proteins (254-256). Active PKC is known to phosphorylate focal adhesion proteins (257-259), regulate focal adhesion formation (260) leading to an increase in the overall number of focal adhesions per cell (253), and promote focal adhesion-dependant processes such as cell adhesion, spreading and migration ((261, 262) reviewed in (263)).

In this study, we identify SH2B1β as a novel focal adhesion protein. We show that SH2B1β localizes to focal adhesions through its SH2 domain, and provide evidence that PKC may regulate SH2B1β focal adhesion localization through phosphorylation of SH2B1β serines 161 and/or 165. In addition, we present evidence that phosphorylation of serines 161 and/or 165 has a profound effect on overall focal adhesion number which may contribute to the ability of SH2B1β to regulate cell motility.

Results

SH2B1β is a novel focal adhesion protein. Our previous studies have implicated SH2B1β in the control of cytoskeletal dynamics by enhancing GH and PDGF-dependent cell ruffling and lamellipodia formation and GH-induced cell motility (112, 132). To gain further insight into the role of SH2B1β in cytoskeletal dynamics and cell motility, GFP-SH2B1β was visualized in 3T3-F442A fibroblasts. Initial experiments revealed GFP-SH2B1β co-localization with the termini of actin filaments in a pattern characteristic of focal adhesion proteins (Fig. 4.2A). To confirm that SH2B1β is localized to focal adhesions, GFP or GFP-SH2B1β-expressing 3T3-F442A fibroblasts were fixed and stained for the focal adhesion markers, vinculin and FAK. GFP-SH2B1β co-localized with both endogenous vinculin (Fig. 4.2B) and endogenous FAK (Fig. 4.2C). GFP-SH2B1β also co-localized with mCherry-vinculin when both proteins were expressed and visualized in living HeLa cells (Fig. 4.2D), indicating that SH2B1β localizes to focal adhesions in both fibroblast and epithelial cell types.

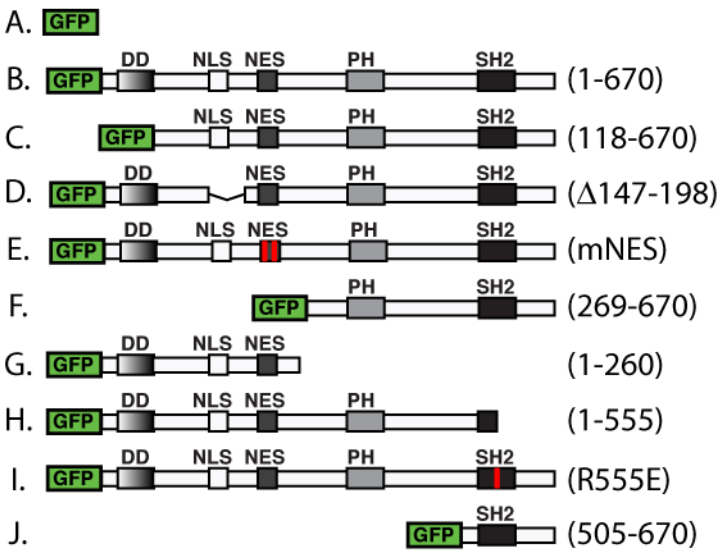

Figure 4.1. Schematic representations of SH2B1β truncation, deletion and point mutations. Red lines indicate point mutations that render the respective domains nonfunctional (107, 248). Schematics are represented in the same order as depicted in Figure 4.3. Abbreviations are: DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain; SH2, Src homology 2 domain.

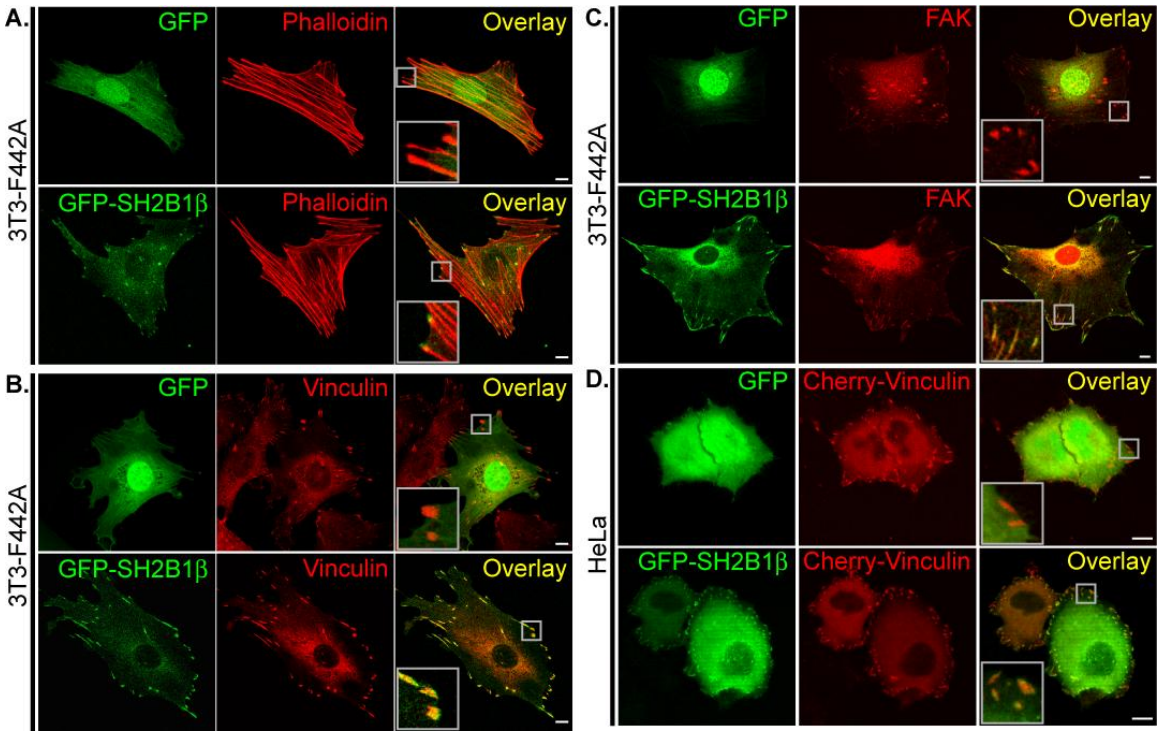

Figure 4.2. SH2B1β localizes to focal adhesions. (A) 3T3-F442A cells expressing GFP (top panel) or GFP-SH2B1β (bottom panel), were fixed and stained with phalloidin to visualize F-actin. (B) 3T3-F442A cells expressing GFP (top panel) or GFP-SH2B1β (bottom panel), were fixed and stained for vinculin to visualize focal adhesions. (C) 3T3-F442A cells expressing GFP (top panel) or GFP-SH2B1β (bottom panel) were fixed and stained for FAK to visualize focal adhesions. (D) HeLa cells expressing GFP and mCherry-vinculin (top panel) or GFP-SH2B1β and mCherry-vinculin (bottom panel) were imaged live. All images were obtained by confocal microscopy. Insets in the overlay images are magnifications of the boxed areas. Scale bar = 10 μm.

The SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization. To determine the region of SH2B1β responsible for focal adhesion localization, a series of GFP-tagged SH2B1β truncation, deletion and point mutants that disrupt SH2B1β signaling domains were co-expressed with mCherry-vinculin in 3T3-F442A fibroblasts (see schematics in Fig. 4.1) and visualized by live confocal microscopy (Fig. 4.3). As expected, GFP-SH2B1β (Fig. 4.3B) but not GFP alone (Fig. 4.3A) localized to focal adhesions. Deletion of the dimerization domain [SH2B1β (118-

670), Fig. 4.3C] or NLS (SH2B1β Δ148-198, Fig. 4.3D) or mutation of the NES (SH2B1β mNES, Fig. 4.3E) did not prevent GFP-SH2B1β from localizating to focal adhesions. GFP-SH2B1β (269-670), which lacks all three of these domains, also retained its ability to localize to focal adhesions (Fig. 4.3F). In contrast, GFP-SH2B1β (1-260), which lacks both the PH and SH2 signaling domains, was unable to localize to focal adhesions (Fig. 4.3G), implicating the PH and/or SH2 domain in SH2B1β focal adhesion localization. GFP-SH2B1β (1-555), which contains the PH domain but lacks an intact SH2 domain, was also unable to localize to focal adhesions (Fig. 4.3H), indicating that the SH2 domain is necessary for focal adhesion localization. GFP-SH2B1β (R555E) lacks a functional SH2 domain due to a single point mutation (248). The inability of this mutant to localize to focal adhesions (Fig. 4.3I) indicates that the SH2 domain alone, and not the region C-terminal to the SH2 domain, is necessary for focal adhesion localization. Finally, GFP-SH2B1β (505-670), which includes primarily the SH2 domain, was able to localize to focal adhesions (Fig. 4.3J), indicating that the SH2 domain is both necessary and sufficient for focal adhesion localization.

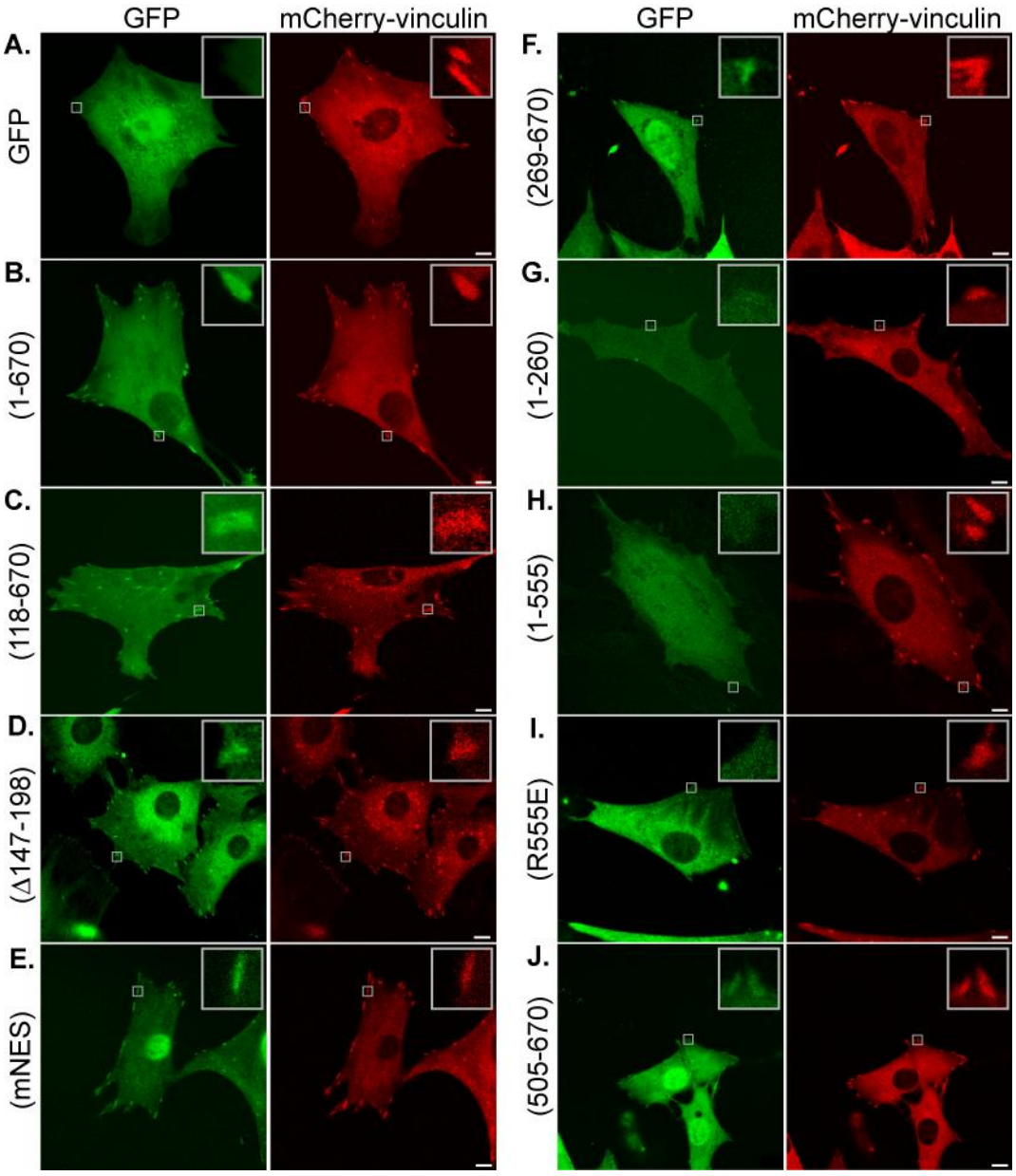

Figure 4.3. The SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization. (A-J) Live 3T3-F442A cells co-expressing mCherry-vinculin with GFP, GFP-SH2B1β or the indicated mutant GFP-SH2B1β were visualized by confocal microscopy. Insets in the overlay images are magnifications of the boxed areas. Scale bar = 10 μm.

Growth hormone stimulation increases SH2B1β turnover dynamics at focal adhesions. Tyrosyl and serine phosphorylation is known to alter the rates at which

proteins cycle into and out of focal adhesions (focal adhesion protein turnover dynamics) (129). GH stimulation induces JAK2-mediated tyrosyl-phosphorylation of SH2B1β in 3T3-F442A cells (100). Therefore, we asked whether GH stimulation alters SH2B1β turnover dynamics in focal adhesions. We assessed GFP-SH2B1β turnover dynamics at focal adhesions using fluorescence recovery after photobleaching (FRAP) (Fig. 4.4). Normalized recovery was substantially increased for GFP-SH2B1β at focal adhesions in cells treated with GH compared to control cells (Fig. 4.4A). While the fraction of mobile GFP-SH2B1β calculated for each condition was similar (Fig. 4.4A, left inset), the *t*1/2, or time need to recover to 50% of the maximum (Fig. 4.4A, right inset), was significantly reduced in GH-treated cells, indicating that GH treatment significantly increases the rate at which SH2B1β cycles in and out of focal adhesions.

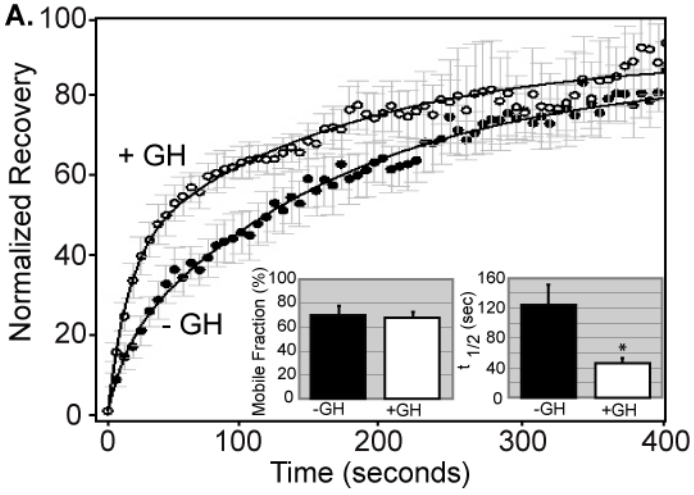

Figure 4.4. GH stimulation increases GFP-SH2B1β turnover dynamics at focal adhesions. 3T3-F442A cells expressing GFP-SH2B1β were incubated in serum-free medium overnight. During the photobleaching scans, cells were treated with (n=5) or without (n=8) 500 ng/ml GH. FRAP analysis was carried out for 400 seconds. FRAP values were obtained using Fluoview software. Curves were fit to normalized data using SigmaPlot software. Mobile fraction (left insets) and $\tau_{1/2}$ (right insets) values were calculated from the curve fit equations. Error bars indicate s.e.m. **P* <0.05 by Student's *t*-test.

PKC mediates PMA-induced SH2B1β phosphorylation. We have previously shown that SH2B1β is serine phosphorylated in response to the PKC agonist, phorbol 12-myristate 13-acetate (PMA), in the PC12 neuronal cell line (110). Our present identification of SH2B1β as a focal adhesion protein combined with the previous observations that several PKC isoforms localize to focal adhesions raise the possibility that PKC regulates SH2B1β at focal adhesions. We first confirmed that SH2B1 is phosphorylated in response to PMA in 3T3-F442A fibroblasts. PMA stimulation activated ERK1/2 (Fig 4.5A, middle panel), consistent with previous studies showing PKC-mediated activation of the Raf/MEK/ERK pathway (264-266). PMA stimulation also caused a significant upward shift in SH2B1 mobility in Western blots (Fig. 4.5A, top panel), an event that we have previously shown to be indicative of increased serine/threonine phosphorylation of SH2B1 (100, 224). Dose and time course experiments revealed PMA stimulation to result in maximal SH2B1β phosphorylation at 100 nm PMA after 15-30 min (Fig. 4.5B). Pre-treating cells with the PKC-specific inhibitor, bisindolylmaleimide I (bis I), but not its inactive analogue, bisindolylmaleimide V (bis V), prior to PMA stimulation inhibited SH2B1 phosphorylation (Fig. 4.5C), indicating that PKC or a kinase downstream of PKC mediates the PMA-induced SH2B1 phosphorylation in 3T3-F442A fibroblasts. Treatment of 3T3-F442A fibroblasts expressing GFP-SH2B1β with PMA also resulted in an upward shift in migration of GFP-SH2B1β (Fig. 4.5D) consistent with exogenously expressed SH2B1β also being phosphorylated in response to PKC activation.

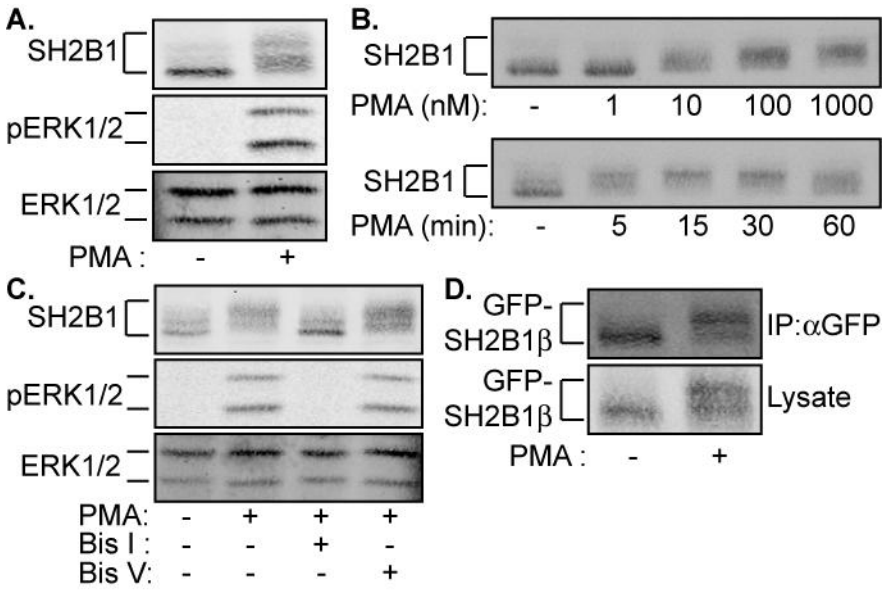

Figure 4.5. PMA induces SH2B1 phosphorylation. (A) 3T3-F442A cells were stimulated with vehicle or 100 nm PMA for 15 min. Lysates were blotted for total SH2B1 (top panel), phospho-ERK1/2 (middle panel) or total ERK1/2 (bottom panel). (B) 3T3-F442A cells were stimulated with increasing concentrations of PMA for 15 min (top panel) or with 100 nm PMA for increasing time periods (bottom panel). Lysates were blotted with αSH2B1. (C) 3T3-F442A cells were pretreated with DMSO, bis I or bis V for 1 hr, then stimulated with vehicle or 100 nm PMA for 15 min. Lysates were blotted with αSH2B1, αphospho-ERK1/2 or αERK 1/2. (D) 3T3-F442A cells expressing GFP-SH2B1β were stimulated with vehicle or 100 nm PMA for 15 minutes. Lysates were immunoprecipitated with αGFP. Immunoprecipitates (top panel) and lysates (bottom panel) were blotted with αGFP.

Phosphorylation of Serines 161/165 regulates localization of SH2B1β in focal adhesions. We have shown previously by mass spectrometry that serine 161 in SH2B1β is phosphorylated and have indirectly implicated phosphorylation of serine 165 in PMA-stimulated 293T cells (Maures, et al. Manuscript in preparation). Both serines lie within the classic PKC-substrate motif, X(S/T)X(R/K) (Center for Biological Sequence Analysis, http://www.cbs.dtu.dk/index.shtml) (Fig. 4.6A). To investigate the potential for phosphorylation of these Ser to regulate SH2B1β at focal adhesions, GFP-SH2B1β, GFP-SH2B1β (S161,165A) (which lacks these two serines) and GFP-SH2B1β (S165E),

(in which Ser 165 has been mutated to glutamate, mimicking phosphorylation) were expressed in 3T3-F442A fibroblasts (Fig. 4.6B). Whereas GFP-SH2B1β localizes to focal adhesions and the cytoplasm, GFP-SH2B1β (S161,165A) localized almost exclusively to focal adhesions. Conversely, GFP-SH2B1β (S165E) localized less to focal adhesions and more to the cytoplasm. Additionally, on average, GFP-SH2B1β (S161,165A)-positive focal adhesions appeared larger than GFP-SH2B1β-positive focal adhesions. These results suggest that phosphorylation of Ser 161 and/or 165 regulates SH2B1β localization at focal adhesions, and possibly the size of focal adhesions.

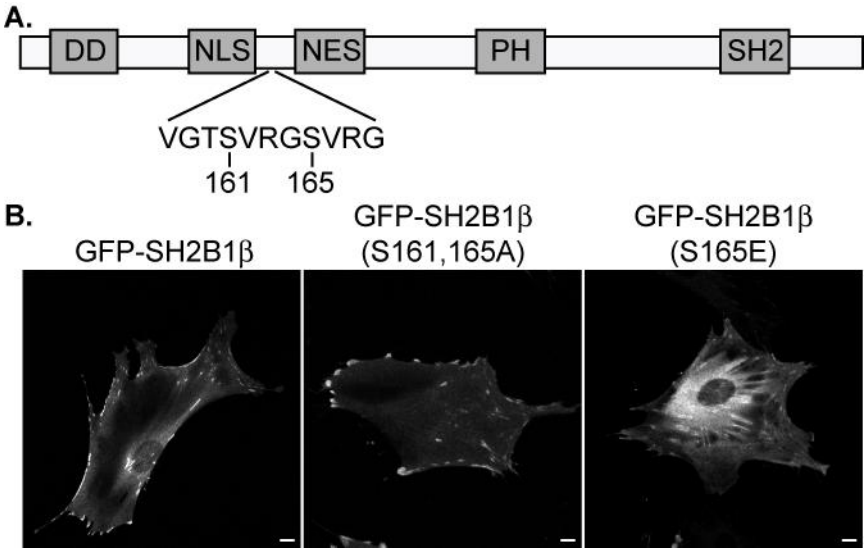

Figure 4.6. Serines 161 and 165 regulate SH2B1β localization within focal adhesions. (A) Schematic representation of serines 161 and 165 in SH2B1β. Abbreviations are: DD, dimerization domain; NLS, nuclear localization sequence; NES, nuclear export sequence; PH, pleckstrin homology domain; SH2, Src homology 2 domain. (B) Live 3T3-F442A cells expressing WT or mutant GFP-SH2B1β were imaged by confocal microscopy. Scale bar = 10 μm.

To determine if PKC activation influences SH2B1β localization at focal adhesions and whether phosphorylation of Ser 161/165 mediates this potential PKC regulation,

3T3-F442A fibroblasts expressing GFP-SH2B1β or GFP-SH2B1β (S161,165A) were stimulated with PMA. While PMA stimulation resulted in a significant upward shift in mobility for GFP-SH2B1β, GFP-SH2B1β (S161,165A) exhibited a minimal upward shift, consistent with PKC inducing phosphorylation of Ser 161 and/or 165 (Fig. 4.7A).

Interestingly, serines 161 and 165 lie within a 30 amino acid stretch of SH2B1β that contains 13 serines and threonines. Ten of these serines and threonines (S137, S141, T142, T143, S144, S145, S154, S157, S161, S165) lie within the classic PKC-substrate motif, X(S/T)X(R/K) (Center for Biological Sequence Analysis, http://www.cbs.dtu.dk/index.shtml). 3T3-F442A cells expressing GFP-vinculin, WT GFP-SH2B1β or GFP-SH2B1β (13SA) (in which all 13 serines and threonines were mutated to alanine) were also imaged by confocal microscopy before and after PMA stimulation. Confocal images were taken every 5 min for 30 min (Fig. 4.7B) and the fluorescence intensity of individual focal adhesions was measured and quantified at each time-point (Fig. 4.7C). PMA stimulation resulted in a significant reduction (> 50%) of GFP-SH2B1β at focal adhesions over 30 min. Mutating Ser 161/165 to alanine substantially reduced the degree of PMA-mediated depletion of GFP-SH2B1β from focal adhesions (to <20%). Interestingly, PMA stimulation of GFP-SH2B1β(13SA) resulted in phenotype almost identical to that of GFP-SH2B1β (S161,165A) (data not shown). PMA stimulation only significantly reduced GFP-vinculin localization at focal adhesions at the 25 min time point, indicating that PMA stimulation does not induce general focal adhesion dissolution. These data suggest that PMA-mediated PKC activation and subsequent SH2B1β phosphorylation at Ser 161 and/or 165 lead to SH2B1β dissociation from focal adhesions.

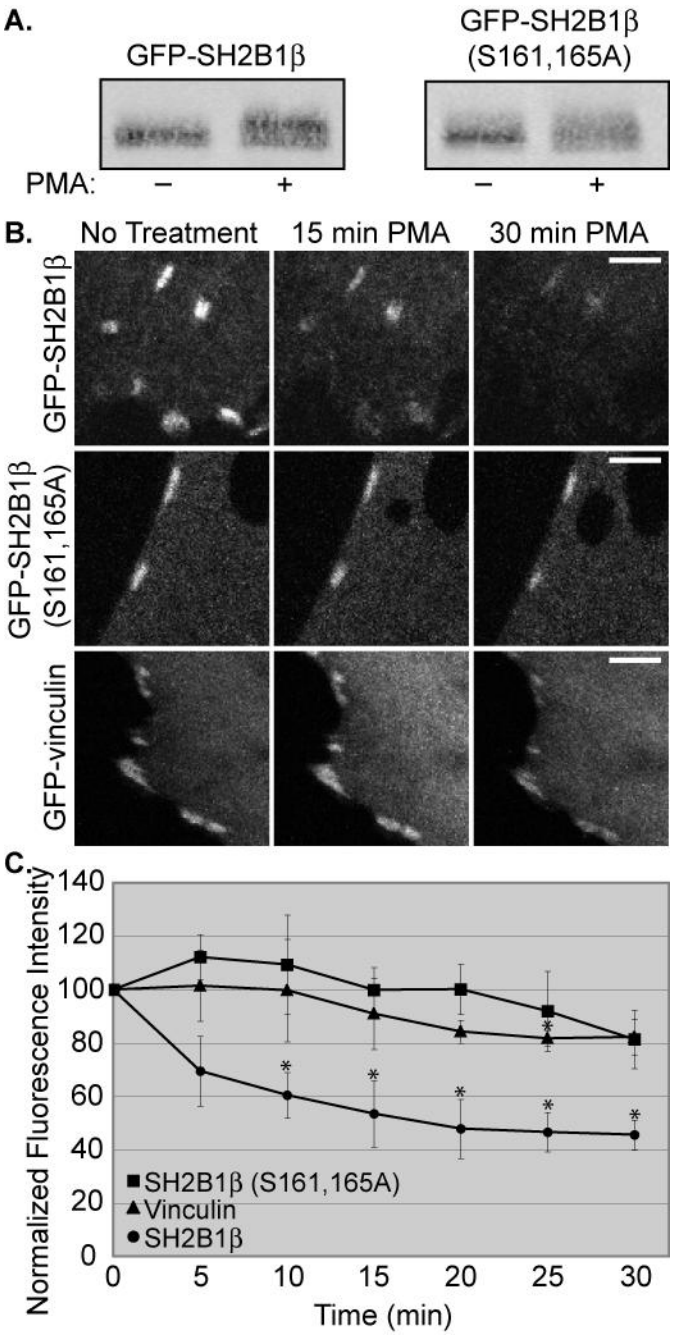

Figure 4.7. PMA stimulation induces a loss of GFP-SH2B1β, but not GFP-SH2B1β (S161,165A), from focal adhesions. (A) 3T3-F442A cells expressing WT or mutant GFP-SH2B1β were stimulated with vehicle or 100 nm PMA for 15 min. Lysates were blotted with αGFP. (B) 3T3-F442A cells expressing GFP-SH2B1β were imaged by confocal microscopy before and after 100 nm PMA stimulation for up to 30 min. (C) Metamorph imaging software was used to quantify the fluorescence intensity of individual focal adhesions. Three independent experiments assessing focal adhesions from 2-4 cells were performed for WT and mutant GFP-SH2B1β and two independent experiments assessing focal adhesions from 2-4 were performed for GFP-vinculin. Error bars indicate s.e.m. **P*<0.05 by Student's *t*-test.

Serines 161/165 regulate SH2B1β focal adhesion dynamics. The observed differences in mutant SH2B1β localization and response to PMA stimulation compared to WT SH2B1β suggest that phosphorylation of Ser 161 and/or 165 modulate SH2B1β turnover dynamics at focal adhesions. To assess the turnover dynamics of focal adhesion-localized SH2B1β, and determine whether phosphorylation of Ser 161 and/or 165 are likely to regulate this turnover, FRAP experiments were performed on focal adhesion-localized GFP-SH2B1β, GFP-SH2B1β (S161,165A) and GFP-SH2B1β (S165E) in 3T3-F442A fibroblasts (Fig. 4.8A). Normalized recovery was substantially delayed for GFP-SH2B1β (S161,165A) and slightly accelerated for GFP-SH2B1β (S165E) compared to WT GFP-SH2B1β (Fig. 4.8B). The fraction of mobile SH2B1β (mobile fraction, Fig. 4.8C) and time needed for SH2B1β to recover 50% fluorescence intensity ($\tau_{1/2}$, Fig. 4.8D) were also determined. While the mobile fraction of both mutants was similar to WT, the $\tau_{1/2}$ of GFP-SH2B1β (S161,165) was significantly increased compared to WT GFP-SH2B1β, and the $\tau_{1/2}$ of GFP-SH2B1β (S165E) appeared to be decreased (although statistical significance was not achieved, $p = .09$) compared to WT GFP-SH2B1β (Fig. 4.8C). Interestingly, GFP-SH2B1β (13SA) again displayed an almost identical phenotype to GFP-SH2B1β (S161,165A) (data not shown). These data indicate that while the total fraction of SH2B1β able to dynamically cycle in and out of focal adhesions is not affected by mutating Ser 161/165, the rate of turnover is significantly decreased when Ser 161 and 165 cannot be phosphorylated. This provides further evidence that phosphorylation of one or both of these residues regulates localization of SH2B1β in focal adhesions.

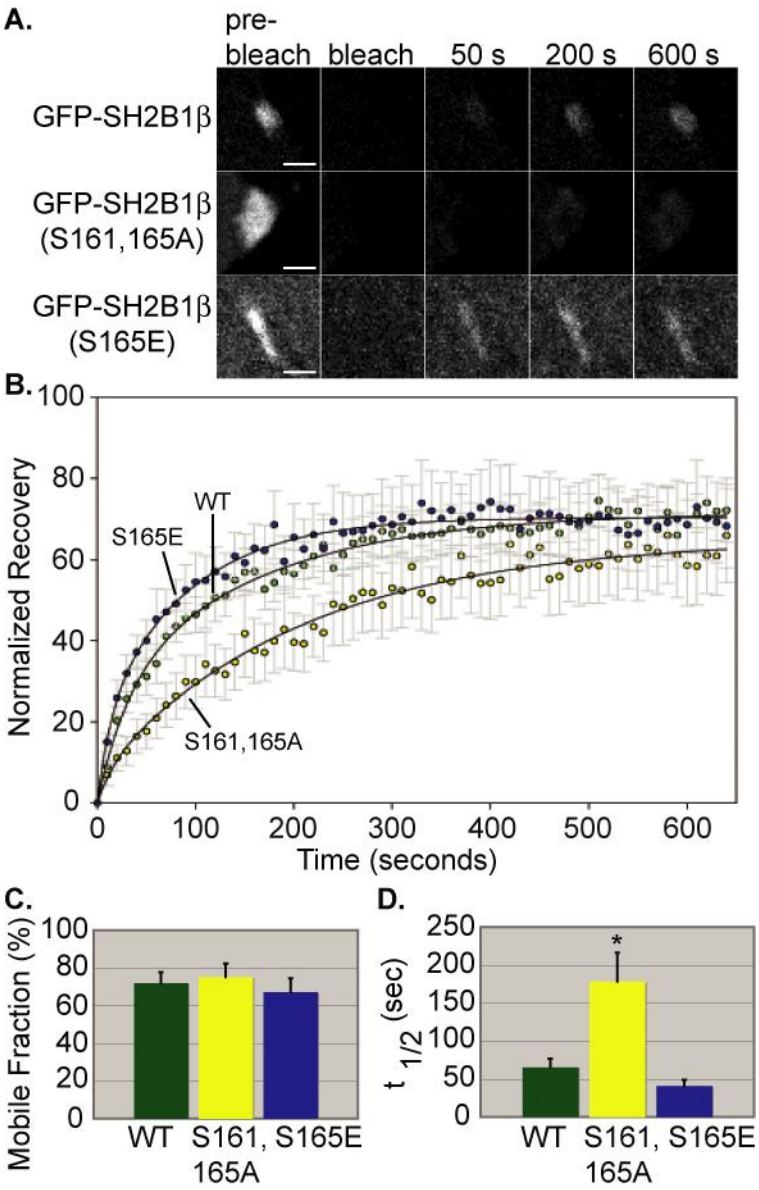

Figure 4.8. Serines 161 and/or 165 regulate SH2B1β focal adhesion dynamics. (A) Confocal microscopy images of focal adhesion-localized WT and mutant GFP-SH2B1β in 3T3-F442A cells before and after photobleaching. Scale bare = 2 μm. (B) FRAP values were obtained using Fluoview software. Curves were fit to normalized data using SigmaPlot software. Mobile fraction (C) and $\tau_{1/2}$ (D) values were calculated from the curve fit equations. n=9 (WT and S161,165A); n=10 (S165E). Error bars indicate s.e.m. **P* <0.01 by Student's *t*-test.

GFP-SH2B1β (S165E) increases focal adhesion number. PKC activation is associated with increased focal adhesion formation (253). Intriguingly, expression of GFP-SH2B1β (S165E) alone also appeared to produce an increased number of focal adhesions per cell as well as smaller focal adhesions compared to cells expressing WT GFP-SH2B1β (Fig. 4.9A.) Quantification of focal adhesions per cell showed that expression of GFP-SH2B1β (S165E) resulted in a significant increase in the number of focal adhesions per cell compared to cells expressing GFP alone, WT GFP-SH2B1β or GFP-SH2B1β (S161,165A) (Fig. 4.9B). This raises the exciting possibility that in addition to regulating focal adhesion-localized SH2B1β turnover, phosphorylation of SH2B1β at Ser 165 is a key event in the PKC-mediated regulation of nascent focal adhesion formation.

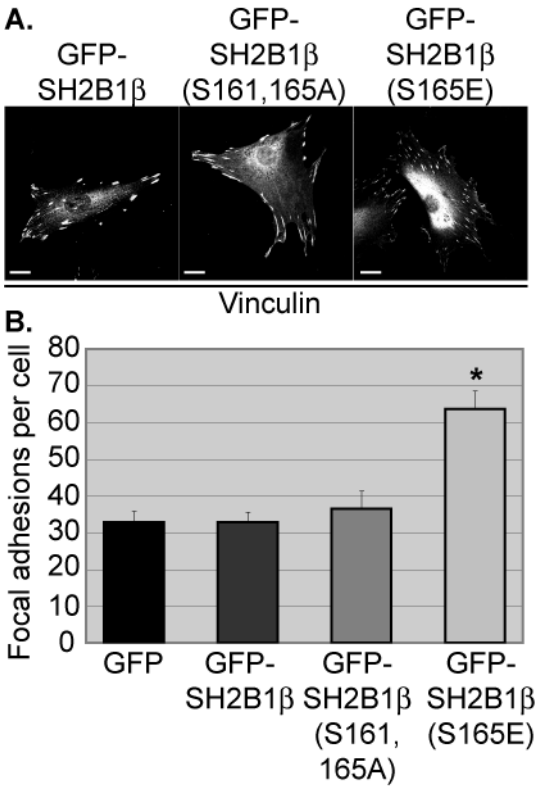

Figure 4.9. SH2B1β (S165E) increases the number of focal adhesions per cell. (A) 3T3-F442A cells expressing WT or mutant GFP-SH2B1β were fixed, stained for vinculin and imaged by confocal microscopy. Scale bar = 10 μm. (B) Quantification of number of focal adhesions per cell. Three independent experiments assessing focal adhesions from at least 10 cells were performed for each condition. Error bars indicate s.e.m. **P* <0.01 by Student's *t*-test.

Discussion

Here, we have identified SH2B1β as a novel focal adhesion protein that is targeted to focal adhesions through its SH2 domain. We further provide evidence that SH2B1β localization at focal adhesions is regulated by PMA-induced phosphorylation, most likely of serines 161 and/or 165, and that this phosphorylation regulates overall focal adhesion number. That the SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization indicates that SH2B1β binds a tyrosyl phosphorylated protein within the focal adhesion complex. Focal adhesions are known to contain several tyrosyl-phosphorylated proteins, including paxillin (267), vinculin (268, 269), talin (270) p130Cas (271), tensin (272), and FAK (267). The SH2 domains of other focal adhesion proteins have been identified as important for focal adhesion localization (273, 274) and overall tyrosine phosphorylation of focal adhesion proteins is thought to be an important regulatory mechanism of focal adhesion formation, stability and maturation (reviewed in (275)). Therefore, in addition to the regulation of SH2B1β focal adhesion localization by PKC activation, it seems likely that mechanisms regulating overall focal adhesion protein tyrosyl phosphorylation (eg. Src and FAK activation) also regulate the recruitment of SH2B1β to focal adhesion. In addition to SH2B1β, we found SH2B1γ and SH2B1δ to localize to focal adhesions (data not shown), and we predict that SH2B1α also localizes to focal adhesions as SH2B1α contains the same SH2 domain as β, γ and δ isoforms. SH2B2 and SH2B3 both contain SH2 domains similar to SH2B1, and all three family members have been shown to bind phospho-Tyr813 in JAK2 through their SH2 domains (22, 23, 276). This suggests that SH2B family members may be recruited to similar targets through their SH2 domains, and therefore it is possible that SH2B2 and SH2B3, like SH2B1, are localized to focal adhesions. SH2B3 has also been described as a

binding partner of the focal adhesion protein, filamin (139), although this interaction was not mediated through the SH2 domain of SH2B3 and not observed at focal adhesions.

It has recently been shown that the rates at which individual proteins cycle into and out of focal adhesions can directly affect the rates of focal adhesion assembly and disassembly (129, 252) as well as cell motility. Therefore, our finding that GH stimulation can directly and significantly regulate the turnover dynamics of a focal adhesion protein (i.e. SH2B1) (Fig. 4.4A) provides one possible mechanism by which GH regulates cell motility. Consequently, it will be of interest in the future to determine the effect that altered rates of SH2B1β turnover dynamics at focal adhesions have on cell motility.

Previous studies have shown that PKC may directly phosphorylate several focal adhesion proteins including vinculin (257), talin (258), filamin (259) and integrin (277). In addition, PKC is an important factor in integrin-mediated signaling (reviewed in (263)) and is able to induce activation of ERK1/2 (253, 278, 279), RhoA (280), and FAK (281), events which lead to an increase in focal adhesion number, cell adhesion and/or cell migration. Our recent work suggests that PKC regulates SH2B1β localization at the plasma membrane (PM) in PC-12 neuronal cells and 293T cells through phosphorylation at Ser 161 and/or 165 (Maures, et al. Manuscript in preparation). These data combined with the data presented in the present study suggest the existence of an intriguing model whereby PKC regulates SH2B1β at multiple levels (ie. at the PM and focal adhesions). Interestingly, several studies have shown that different PKC family members are localized to distinct subcellular locations including the PM and focal adhesions (reviewed in (282)), raising the possibility that specific PKC family members regulate SH2B1β at different subcellular locations. In the PC-12 model, it is thought that positively charged amino acids within the NLS recruit SH2B1β to the negatively charged PM. The negative

charge from phosphorylation of Ser 161/165 (which lie adjacent to the positively charged NLS) counteracts this positive charge, leading to dissociation of SH2B1β from the PM. Here, we show that the NLS is dispensable for SH2B1β focal adhesion localization (Fig. 4.3), indicating that phosphorylation of Ser 161/165 causes SH2B1β dissociation from focal adhesions by some other mechanism (e.g. a phosphorylation-induced conformational change leading to a decreased affinity of SH2B1β for a binding partner). Precedence exists for serine phosphorylation-mediated regulation of focal adhesion protein localization. Serine phosphorylation of vinculin (283) and paxillin (284) is known to cause dissociation of these molecules from focal adhesions. Serine phosphorylation of paxillin has also been shown to regulate paxillin dynamic turnover at focal adhesions (129). Inhibiting Ser 161/165 phosphorylation or mimicking constitutive Ser 165 phosphorylation similarly altered SH2B1β turnover dynamics at focal adhesions, providing further evidence that regulated phosphorylation at these sites is important for SH2B1β function at focal adhesions.

Our surprising finding that focal adhesion numbers per cell were significantly increased by mimicking Ser 165 phosphorylation by mutation to Glu suggests that phosphorylation of SH2B1β at Ser 165 may be a key step in the formation of new focal adhesions. In addition, cells expressing SH2B1β (S165E) appeared to harbor smaller focal adhesions than cells expressing GFP or WT GFP-SH2B1β (Fig. 4.9 and data not shown). Interestingly, FAK null fibroblasts also display increased numbers of focal adhesions compared to WT cells (285) and vinculin null fibroblasts display focal adhesions that are smaller in size (250). However FAK null fibroblasts are less motile than their WT counterparts while vinculin null fibroblasts are more motile than WT cells but have a decreased number of focal adhesions per cell. SH2B1β (S165E) appears to incorporate aspects of both of these opposing phenotypes as we have also found

SH2B1β (S165E) to potently increase cell migration (HWS paper}. Perhaps the phenotype that most closely resembles that of SH2B1β (S165E) is that of PMA-induced PKCε activation in glioma cells, which results in both an increase in focal adhesion number and cell migration (253).

One mechanism by which SH2B1β may regulate focal adhesion number is by modulating the dynamic turnover of other focal adhesion proteins. There is evidence indicating that turnover of individual focal adhesion proteins may be directly related to the turnover of focal adhesions themselves (252), a process that is linked to the formation of new focal adhesions. After localizing to focal adhesions through its SH2 domain, SH2B1β may bind and stabilize other focal adhesion proteins through its other signaling domains, thus providing additional integrity to the focal adhesion complex. In addition, cells expressing GFP-SH2B1β (S161,165A) appeared to harbor large focal adhesions with many fibrillar adhesions (Fig. 4.9 and data not shown) suggesting that focal adhesions in GFP-SH2B1β (S161,165A)-expressing cells either mature more rapidly or turn over less rapidly than in cells expressing WT GFP-SH2B1β. Taken together, these observations suggest that PKC-mediated phosphorylation of SH2B1β and subsequent redistribution out of focal adhesions may initiate partial destabilization of focal adhesions, leading to smaller and increased number of focal adhesions, resulting in increased cell motility.

The regulation of cell signaling, cytoskeletal dynamics and cell motility through focal adhesions is crucial for numerous physiological and pathophysiological processes. A growing number of studies also implicate SH2B family members in an array of physiological processes that are dependent on cytoskeletal dynamics and/or cell motility. Our finding of SH2B1β as a novel focal adhesion protein whose phosphorylation

dynamically regulates focal adhesion number provides the grounds for further study into the precise function and regulation of SH2B1β in focal adhesions.

MATERIALS AND METHODS

Antibodies: Anti-vinculin mouse monoclonal antibody (α-vinculin) was from Sigma-Aldrich (Cat. # V9131) and anti-FAK mouse monoclonal antibody (α-FAK) was from Transduction Laboratories (Cat. # F15020). Both were used at a dilution of 1:100 for immunofluorescence. Polyclonal antibody to rat SH2B1 {(αSH2B1), kind gift of Dr. Liangyou Rui (University of Michigan)}, was raised against an SH2B1β glutathione S-transferase fusion protein and used at a dilution of 1:1000 for Western blotting (243). Anti-phospho-44/42 MAPK antibody that recognizes both ERK1 and ERK2 that are doubly phosphorylated on T202/Y204 (αpERK1/2; E10) and anti-total ERK (αERK1/2) were from Cell Signaling Technology and were used at a dilution of 1:1000 for Western blotting. IRDye 800- and IRDye 700-conjugated affinity-purified antimouse IgG and antirabbit IgG (Rockland Immunochemicals Inc.) were used at a dilution of 1:20,000. Anti-GFP IRDye 800-congujated goat polyclonal antibody (Rockland) was used at a dilution of 1:5000 for Western blotting. Anti-GFP mouse monoclonal antibody (αGFP) for immunoprecipitation (1:100 dilution) was from Clontech. Alexa Fluor 568-conjugated phalloidin (dilution 1:100) and anti-mouse Alexa Fluor 405 and 594 secondary antibodies (dilution 1:1000) for confocal immunofluorescence were from Invitrogen.

Reagents: Recombinant 22,000-Da human GH was a kind gift from Eli Lilly & Co. PMA (Sigma) was diluted in DMSO. Dulbecco's Modified Eagle Medium (DMEM) was from Cambrex. Fetal bovine serum (FBS) was from Hyclone. Calf serum was from Atlanta Biologicals. The antibiotic-antimycotic solution, trypsin-EDTA and Magic Mark XP western standards were from Invitrogen. Aprotinin, leupeptin, and Triton X-100 were from Roche. Recombinant protein A-agarose was from Repligen. Hybond-C Extra nitrocellulose was from Amersham Biosciences. Bisindolylmaleimide I and bisindolylmaleimide V were from

Calbiochem. Human fibronectin was from BD Biosciences. Paraformaldehyde was from Electron Microscopy Sciences.

Cell Culture, Transfection and Stimulation: The stock of murine 3T3-F442A fibroblasts was kindly provided by H. Green (Harvard University). HeLa cells were from ATCC. 3T3-F442A cells were grown in DMEM supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 8% calf serum. HeLa cells were grown in DMEM supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 10% fetal bovine serum. 3T3-F442A fibroblasts were transiently transfected using Amaxa nucleofector technology from Lonza (Colonge, Germany) using solution V and setting U24. HeLa cells were transiently transfected using FuGene HD from Roche (Indianapolis, IN). All cells were incubated overnight in serum-free medium containing 1% bovine serum albumin before treatment with PMA or GH.

Plasmids, Cloning and Mutagenesis: All cDNAs encoding GFP-tagged SH2B1β and SH2B1β point, deletion and truncation mutants were described previously (106, 107, 110, 112, 132) (Maures, et al. Manuscript in preparation). GFP-vinculin was a kind gift from Dr. Kenneth Yamada (National Institutes of Health).

Immunofluorescence: For fixed-cell imaging cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), gently washed 3X in PBS, permeabilized in 0.1% TritonX-100 in PBS, blocked for 30 minutes in PBS containing 5% normal serum from the species used for secondary antibody production. Cells were then incubated with primary antibody diluted 1:100 in blocking solution for one hour. Cells were gently washed 3X in PBS, and then incubated for 1 h with secondary antibody diluted 1:1000 in PBS or alexa fluor-conjugated phalloidin diluted 1:100 in PBS. Cells were gently washed 3X in ddH_2O, and then mounted on Fisherfinest Premium

Microscope Slides using Prolong Gold Antifade mounting reagent (Invitrogen). For live cell imaging, cells were grown on no. 1.5 glass bottom dishes (MatTek Corp.) and imaged in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ $6H_2O$, 2 mM NaH_2PO_4 H_2O, 10 mM HEPES, 10 mM glucose). All images were obtained with an Olympus FluoView 500 Laser Scanning Confocal Microscope. For focal adhesion counting, cells were fixed and stained for vinculin. 15-20 random images were obtained in each of three independent experiments for each condition, and vinculin-positive focal adhesions in each cell were counted.

Fluorescence Recovery After Photobleaching: GFP-SH2B1β, GFP-SH2B1β (S161,165A) or GFP-SH2B1β (S165E) were transiently expressed in 3T3-F442A fibroblasts. The GFP signal in individual focal adhesions was bleached by 3 iterations of 100% laser power. Fluorescent intensity measurements were taken every 6 or 10 sec. Data were normalized to unbleached sections of cytosol after background subtraction. SigmaPlot 11.0 was used to fit curves to FRAP data by applying the nonlinear regression of exponential rise to maximum and the double fours best fit equation, $y = a*(1–e^{–b*x})+c*(1– e^{–d*x})$. The fraction of mobile SH2B1β and τ1/2 (time needed to recover to 50% of the mobile fraction) were calculated from the equations..

This Chapter represents a “manuscript in progress” and will be submitted for publication under the title “Identification and characterization of SH2B1β as a novel focal adhesion protein” by Nathan J. Lanning, James Herrington, Travis J. Maures, and Christin Carter-Su. James Herrington made the initial observations that SH2B1β is a focal adhesion protein and that the SH2 domain was necessary for focal adhesion localization, Travis J. Maures cloned many of the GFP-SH2B1β mutants used in these studies, and I provided all figures for this Chapter

Chapter 5

Conclusion

The overall goal of my thesis research was to answer some of the important remaining questions in GH signal transduction. In particular, I wanted to identify the primary kinase(s) responsible for transducing the GH signal in 3T3-F442A and H4IIE cells and then more fully characterize the role of SH2B1β in GH-induced regulation of the actin cytoskeleton. While JAK2 and Src family kinases have both been shown to function within the GH signaling pathway, the relative contributions of each to GH signaling in the frequently used models of GH signaling, 3T3-F442A preadipocytes and H4IIE hepatoma cells, remained unclear. The interpretation and application of dozens of previous studies along with the design of future studies utilizing these cell lines rely on the answer to this question. Additionally, many GH-induced responses such as differentiation, proliferation and migration require regulation of the actin cytoskeleton, yet few studies have probed the molecular mechanisms of these actions. From the few studies attempting to identify these mechanisms, SH2B1β has clearly emerged as an important modulator of GH-induced cytoskeletal regulation.

JAK2, but not Src family kinases, regulate Stat, ERK1/2 and PI3-kinase activation in 3T3-F442A preadipocytes and H4IIE hepatoma cells

The results presented in Chapter 2 collectively indicate that JAK2 is primarily responsible for GH activation of Stat1, Stat3, Stat5, ERK1/2 and Akt in 3T3-F442A fibroblasts, H4IIE hepatoma cells and murine embryonic fibroblasts (MEFs). While the overall interpretation of the results in Chapter 2 is fairly straight forward, there are instances where further discussion is warranted. Prior to the study described in Chapter 2, JAK2 had been classically thought of as the primary kinase responsible for transducing the GH signal (5). However, in recent years, several reports have surfaced indicating that, like other members of the cytokine family, GH also activated members of the Src family of kinases (these reports are described in detail in the Introduction section of this thesis). Like JAK2, Src family kinases are able to induce the activation of Stat, ERK1/2 and Akt proteins (reviewed in (286)) raising the possibility that GH activation of Src family kinases may contribute to some GH signaling events. Therefore, we sought to determine the relative contribution of JAK2 and Src family kinases to GH signaling in two highly GH responsive cell types: 3T3-F442A preadipocytes and H4IIE hepatoma cells. 3T3-F442A preadipocytes require GH in order to mature into adipocytes (168). GH has also been shown to regulate the actin cytoskeleton in these cells (132, 169). In differentiated 3T3-F442A adipocytes, GH regulates the transcription of multiple genes (172), as well as lipolysis, hormone-sensitive lipase (170) and rates of glucose transport (171). Hepatocytes are a major target of GH-mediated transcriptional regulation (reviewed in (39)), and H4IIE hepatoma cells have been used to study the effect of GH on protein synthesis (180) and insulin responsiveness (179, 181, 182). Therefore, we sought to determine the relative contribution of the aforementioned kinases to GH signaling in these cells.

Src family kinases were not found to be activated in response to GH in either of these cell lines. In data not included in Chapter 2, we were able to identify by molecular weight the active forms of all six Src family kinases present in 3T3-F442A and H4IIE cells, indicating that the method that we chose to assess Src family kinase activity (i.e. blotting with a phospho-specific antibody specific to the activating tyrosine) did not overlook activation of any Src family kinase members. Additionally, as described in Chapter 2, we found EGF stimulation of 3T3-F442A cells to significantly further activate Src family kinases. Thus Src family kinases were not maximally activated in the basal state, and were capable further activated by physiologically relevant ligands. Pretreatment with Src family kinase pharmacological inhibitors reduced the activity of all six Src family kinases to levels below detection. However, the inhibitors had no effect on GH-induced activation of JAK2, ERK1/2, Stat1, Stat3, or Stat5. Collectively, these results showed that in 3T3-F442A and H4IIE cells, GH-induced activation of JAK2, ERK1/2 and Stat proteins is not dependent on Src family kinase activity. We cannot rule out the possibility that Src family kinases account for a fraction of GH activation of these proteins that is too small to be detected by this established method of Src family kinase activation detection. However, we hypothesize that any such contributions by Src family kinases to the physiological actions of GH in these cells would be negligible. In addition, these results argue against the possibility that GH treatment results in the recruitment of basally active Src family kinases to a cellular compartment where they can then activate ERKs and Stats.

Pharmacological inhibition of Src family kinases did, however, result in a significant reduction in GH-induced Akt activation in both cell lines. However, the inhibitors also significantly reduced basal Akt activity, raising the possibility that the Src inhibitor-induced reduction in GH-induced Akt activation was not specific to GH-induced activation, but was instead related to decreased basal Akt activity. One group has

proposed that Src must phosphorylate Akt on Tyr315 and Tyr326 before growth factors can activate Akt by stimulating its phosphorylation on Thr308 and Ser473 (201, 202). Thus, while Src phosphorylation of Akt may be necessary for GH to induce Akt activation, Src may not directly mediate that activation. In support of this, we found that in 3T3-F442A cells pretreated with Src family kinase inhibitors, epidermal growth factor was, like GH, unable to activate Akt (data not shown). Further, we found that reducing JAK2 levels by RNAi almost abolished GH-induced activation of Akt, and that GH was able to substantially activate Akt in Src family kinase null fibroblasts. These data indicate that GH is able to activate Akt without Src family kinases, but seems unable to activate Akt without JAK2. Additionally, PP3, the inactive analogue of the Src family kinase inhibitors, PP1 and PP2, inhibited both basal and GH-stimulated Akt activation. This raises the additional possibility that the inhibitory effects of the active Src family kinase inhibitors, PP1 and PP2, are not mediated exclusively via Src family kinases, but rather have off-target effects as has been previously documented (189, 190).

The overall results of our study are in direct opposition to those generated by the Lobie, Tanaka, and Waters groups who found GH to activate at least one Src family kinase in CHO cells, NIH 3T3 cells, F-36P human leukemia cells, FDC-P1 hematopoietic progenitor cells, or murine liver tissue (30, 32, 34, 287). The simplest explanation for this apparent discrepancy is that different cell types activate different kinases in response to GH. For example, cells of hematopoietic origin appear to utilize Src family kinases for cytokine signaling to a greater extent than cells of non-hematopoietic origin (165). However, the reason why different cell types utilize different kinases for signal transduction remains unknown. One hypothesis is that different cell types express different relative amounts of particular kinases. In a situation in which two kinases are able to transduce the same signal, the relative amount of one kinase compared to another will determine which kinase is used (i.e. if there are more JAK2 molecules than

Src family kinase molecules, JAK2 will transduce the GH signal). This explanation may be satisfactory for the example of F-36P and FDC-P1 cells compared to 3T3-F442A and H4IIE cells as F-36P and FDC-P1 cells are of hematopoietic origin, and thus may have higher levels of Src family kinases compared to JAK2 than fibroblasts and hepatoma cells. However, this explanation does not seem to explain the differences in results between our 3T3-F442A and MEF cells (derived from three separate mice) and those obtained by the Lobie group who used NIH 3T3 cells. All of these cells are derived from mouse fibroblasts, and therefore might be expected to contain similar relative levels of JAK2 and Src family kinases. An alternative explanation for the differences between our results and those of the Lobie group relates to the method of assessing Src activation. We relied on a phospho-specific antibody that detects phosphorylated tyrosine 416 in c-Src and analogous tyrosines in other Src family kinases as an indication of SFK activity. Although the Lobie group relied on analogous phospho-specific antibodies to assess JAK2 and ERK1/2 activation, they utilized an *in vitro* kinase assay to detect GH-activated Src. In this assay, Src was immunoprecipitated with a polyclonal antibody (from Santa Cruz Biotechnology, Inc.) from GH-treated cells, then incubated with a Src substrate in the presence of [γ^{32}-P]ATP (30). We attempted to use a Src polyclonal antibody from Santa Cruz Biotechnology, Inc. for detection of total Src but found that there were too many non-specific bands present on Western blots to identify c-Src or other Src family kinases. While it is possible that when the Lobie group used this antibody for immunoprecipitations and subsequent *in vitro* kinase assays, a relatively pure Src immunoprecipitate was obtained, controls assessing the efficiency of these immunoprecipitations were not shown, leaving questions regarding the specificity of these results.

More recently, the Waters group has reported GH activation of Src in mouse liver tissues (34, 35). This is in seeming contrast with our H4IIE cells, which are derived from

a rat hepatoma. In their animal studies, the Waters group specifically tested GH-induced responses in liver tissue to implicate Src in the activation of ERK, and JAK2 in the activation of Stat5, Stat3 and Akt. These studies were conducted on mice harboring a GHR knock-in that contained proline to alanine mutations within the Box 1 region of the GHR. These point mutations disrupt the ability of JAK2 to bind GHR and, therefore, the ability of GH to activate JAK2. In their data using liver from mutant mice, GH clearly activates Src. Additionally, while JAK2, Stat5 and Akt are not activated in these experiments, ERK1/2 is activated, indicating that GH can activate ERK1/2 in the absence of JAK2 activation. This finding raises the possibility that JAK2 is responsible for GH activation of Stats and Akt in liver tissue while Src is responsible for GH activation of ERK1/2. However, while the two papers characterizing these mutant mice (34, 35) both assert that GH induces Src activation in WT mice, there are no convincing data to substantiate these claims. This raises the possibility that GH is only able to activate Src in the mutant mice due to the fact that JAK2 is unable to be activated. If this is true, this mouse model of GH-induced Src activation may be an artifact of an inability to activate JAK2.

Future Directions

Because GH-mediated activation of Stat, ERK and Akt proteins in 3T3-F442A and H4IIE cells appears to be dependent on JAK2, it would be interesting to determine whether JAK2 is required for more downstream actions of GH. GH is necessary for 3T3-F442A preadipocyte differentiation into mature adipocytes (168). Thus, testing the ability of JAK2 shRNA expressing-3T3-F442A preadipocytes to mature into adipocytes is a logical future experiment. Likewise, assessing the ability of GH to regulate lipolysis, hormone-sensitive lipase and glucose transport rates in JAK2 knockdown 3T3-F442A adipocytes would shed light on the importance of JAK2 to these previously described actions of GH in 3T3-F442A adipocytes (170, 171). H4IIE cells have been used to study the effect of GH on protein

synthesis (180) and insulin responsiveness (179, 181, 182), and therefore repeating these experiments with H4IIE JAK2 knockdown cells would clarify the importance of JAK2 to these GH-induced actions.

Perhaps the most outstanding question stemming from this study is why our study identified JAk2 as the primary upstream kinase in GH signaling while other groups report Src family kinases playing a prominent role in GH signaling. We have hypothesized that cell type and relative kinase abundance may account for these differences. Others have proffered this hypothesis too (35). Future experiments comparing relative levels of JAK2 and Src family kinases in 3T3-F442A, H4IIE, NIH 3T3, CHO, F-36P and FDC-P1 cells are a logical first step to answering this question. If our hypothesis is true, I would expect to observe significantly higher levels of JAK2 relative to levels of Src family kinases in 3T3-F442A and H4IIE cells than in NIH 3T3, CHO F-36P and FDC-P1 cells and mouse liver tissue.

Growth hormone induces SH2B1β and JAK2 to form a complex with the novel SH2B1β-interacting partner, βII-spectrin, and induces βII-spectrin re-localization

In Chapter 3, the relationship of SH2B1β to GH-mediated regulation of the actin cytoskeleton was investigated by characterizing the novel interaction between SH2B1β and the cytoskeleton protein, βIIΣ1-spectrin. At the time this study was undertaken, Rac had previously been identified as an SH2B1β-binding protein, providing one mechanism whereby SH2B1β could modulate GH-induced changes in the actin cytoskeleton {e.g. recruitment of Rac to the actin cytoskeleton via plasma membrane-localized and/or JAK2 associated SH2B1β) {Diakonova, 2002 #3497}. However, in a yeast 2-hybrid screen for novel SH2B1β interacting proteins, the C-terminus of βIIΣ1-spectrin was identified. The importance of spectrins to the regulation and function of the actin

cytoskeleton is well established. Thus, its binding to SH2B1 raised the possibility of an added mechanism whereby SH2B1β is able to mediate or modulate GH-induced changes in the actin cytoskeleton. Subsequent co-immunoprecipitation experiments identified amino acids 2200-2358 of βIIΣ1-spectrin as interacting with amino acids 105-150 of SH2B1β in a mammalian expression system. In data not shown, the SH2B1β region of interaction was further refined to amino acids 118-150. While amino acids 2200-2358 include 97% (102 out of 111 amino acids) of βIIΣ1-spectrin's PH domain, no functional domains are known to lie within amino acids 118-150 of SH2B1β, raising the possibility of a previously undefined PH domain-interacting region in SH2B1β. Alternatively, it is possible that the PH domain of βIIΣ1-spectrin does not mediate the interaction with SH2B1β, but that the 55 amino acids C-terminal to the PH domain are responsible for the interaction. Like the region of interaction within SH2B1β, amino acids 2304-2358 of βIIΣ1-spectrin do not contain any known functional domains.

Interestingly, the extreme N- and C-terminal domains of βIIΣ1- and βIIΣ2-spectrin share no sequence commonality, and βIIΣ1-spectrin amino acids 2200-2358 largely consists of almost the entire C-terminal region of dissimilarity between these splice variants. Therefore, I predicted that βIIΣ2-spectrin will not interact with SH2B1β. In data not shown, immunoprecipitation experiments were performed on lysates from 293T cells expressing full-length βIIΣ2-spectrin and SH2B1β. In these experiments, GFP-SH2B1β was not found to co-immunoprecipitate with myc-βIIΣ2-spectrin, seemingly verifying the hypothesis that SH2B1β binds βIIΣ1- but not βIIΣ2-spectrin. However, it must be noted that these experiments were performed with full-length βIIΣ2-spectrin. In fact, when the same experiment was repeated with full-length βIIΣ1-spectrin, I found that full-length βIIΣ1-spectrin also failed to co-immunoprecipitate with SH2B1β. Further, βIIΣ1-spectrin

(2165-2358), which contains an intact PH domain, also failed to co-immunoprecipitate with SH2B1β in similar experiments. In summary, βIIΣ1-spectrin (2200-2358) consistently co-immunoprecipitates with SH2B1β, while βIIΣ1-spectrin (2165-2358), full-length βIIΣ1-spectrin and full length βIIΣ2-spectrin all fail to co-immunoprecipitate with SH2B1β when exogenously expressed in 293T cells.

The simplest explanation for these apparent discrepancies takes into account the fact that βIIΣ1-spectrin (2200-2358) is localized to the cytoplasm and nucleus while the other spectrins are tightly associated with the plasma membrane (see Figs. 3.4 and 3.7). Spectrins that are tightly associated with the plasma membrane have a high likelihood of remaining in the insoluble fraction of cell lysates, and therefore remaining unavailable for co-immunoprecipitation. On the other hand, a large percentage of βIIΣ1-spectrin (2200-2358) is present in the cytoplasm, and is thus available for co-immunoprecipitation with SH2B1β. All of these co-immunoprecipitation experiments were performed under conditions using lysis buffer containing 0.1% TritonX-100. In the 3T3-F442A model that revealed a GH-induced complex formation between endogenous βII-spectrin, SH2B1β and JAK2, 1% TritonX-100 lysis buffer was used. The more powerful 1% TritonX-100 lysis buffer may account for the positive results observed in the latter experiment.

In the overexpression co-immunoprecipitation and confocal microscopy experiments, βIIΣ1-spectrin (2200-2358) and SH2B1β appeared to interact constitutively. In the co-immunoprecipitation experiment assessing endogenous proteins, a low level of interaction was present in the serum-deprived condition and the level of interaction was significantly increased with GH stimulation. Therefore, in the endogenous system, GH-stimulation (or possibly other ligand stimulation) appears to be necessary for the robust interaction between SH2B1 and βII-spectrin. GH and other ligands are present in serum, providing a possible explanation for the constitutive nature of the interaction in

the overexpression systems. An alternative explanation is that βIIΣ1-spectrin (2200-2358) lacks a region that negatively regulates the interaction with SH2B1β, allowing for constitutive interaction. Further insight into a possible mechanism for this interaction may be gleaned from the facts that co-expression of JAK2 with both full-length spectrin isoforms resulted in tyrosyl phosphorylation of both spectrins, and that GH induced an increase in the interaction between βII-spectrin and SH2B1. Therefore, it is possible that in the basal state, a lower affinity interaction exists between amino acids 2200-2358 of βIIΣ1-spectrin and amino acids 118-150 of SH2B1β. Upon GH stimulation, the SH2B1β/βIIΣ1-spectrin complex is recruited to JAK2, resulting in tyrosyl-phosphorylation of βIIΣ1-spectrin by JAK2, thereby creating higher-affinity binding sites for SH2B1β's SH2 domain. Alternatively, JAK2 phosphorylation of βIIΣ1-spectrin alters its conformation, making amino acids 2200-2358 more readily accessible to SH2B1β.

Notice that this model provides for a direct interaction only between βIIΣ1-spectrin, but not βIIΣ2-spectrin (βIIΣ2-spectrin does not contain amino acids 2200-2358 of βIIΣ1-spectrin). It is possible that a yet undiscovered site of SH2B1β interaction is present on βIIΣ2-spectrin. It is also possible that βIIΣ2-spectrin is present in a heterotetramer with βIIΣ1-spectrin and is co-recruited to JAK2 with βIIΣ1-spectrin, thus allowing for phosphorylation by JAK2.

Interestingly, we found that JAK2 does not phosphorylate amino acids 2200-2358 of βIIΣ1-spectrin (data not shown), which is one of the regions of difference between the splice variants. Thus it seems likely that JAK2 phosphorylates βIIΣ1- and βIIΣ2-spectrin on the same sites. Finally, all identified metazoan spectrins share 50-60% amino acid sequence similarity, with some regions sharing 70-80% sequence similarity (217). If JAK2 phosphorylates βIIΣ1- and βIIΣ2-spectrin within these regions of homology between all spectrins, it is possible that JAK2-mediated phosphorylation of spectrin

repeat-containing proteins is a common and major mode of regulation of the cytoskeleton.

The functional significance of JAK2-mediated phosphorylation of βII-spectrin is yet to be established. Phosphorylation of spectrins is thought to increase their ability to bind actin (218), induce their re-localization from the plasma membrane into the cytoplasm and/or nucleus (219, 221), decrease their affinity for other spectrins (220), decrease their affinity for the plasma membrane (222) and induce their cleavage by proteases (237). Insulin receptor (IR) and epidermal growth factor receptor (EGFR) have both been shown to directly tyrosyl phosphorylate β-spectrin in *in vitro* kinase assays (238, 239). However, these assays were used to assess the relative kinase activities of IR and EGFR and did not link the phosphorylation of β-spectrin with a function. With the exception of tyrosyl-phosphorylation-induced calpain cleavage of αII-spectrin, all known spectrin phosphorylation events linked to a function are induced by serine/threonine phosphorylation of spectrin. This raises the question of whether the GH-induced subcellular redistribution of βIIΣ2-spectrin that we observed in H4IIE cells is directly linked to JAK2-mediated tyrosyl-phosphorylation of βIIΣ2-spectrin or indirectly linked via serine/threonine phosphorylation of βIIΣ2-spectrin by some kinase downstream of JAK2 (e.g. ERK1/2).

One mechanism whereby GH may induce tyrosyl-phosphorylation and subsequent subcellular redistribution of βII-spectrin is through GH stimulation inducing the formation of a complex of βII-spectrin, SH2B1β and JAK2. We observed GH treatment to induce the formation of this complex in 3T3-F442A cells. However, we did not observe tyrosyl phosphorylation of βII-spectrin at the 20 min time point tested. One explanation for the apparent discrepancies between this result and the ability of JAK2 to phosphorylate βII-spectrin in the 239T cell overexpression system is that GH induces βII-

spectrin phosphorylation at a time point prior to or after 20 min. This is a possibility that can be addressed by performing time courses of GH treatment. Alternatively, the anti-phospho-tyrosine antibody used in this experiment may not have been sensitive enough to identify GH-induced phosphorylation of endogenous spectrin, or βII-spectrin may not be tyrosyl phosphorylated in response to GH treatment. Future studies are required to determine if βII-spectrin is phosphorylated in response to GH treatment.

Dissociation of spectrins from the plasma membrane is thought to be an event that precedes proliferation (221). Therefore, JAK2-mediated phosphorylation of spectrin and subsequent dissociation of spectrin from the plasma membrane may be a mechanism whereby GH can induce proliferation. Autocrine GH signaling has recently been implicated in several cancer-associated phenotypes including enhanced proliferation of MCF-7 cells (288). Accordingly, it is possible that GH-induced redistribution of βII-spectrin may contribute to the proliferative actions of GH in MCF-7 cells. Another example where JAK2 phosphorylation of β-spectrins may contribute to proliferative disorders may be in the case of the constitutively active JAK2 mutant, JAK2 V617F, which drives several myeloproliferative disorders, including polycythemia vera, essential thrombocythaemia, and myelofibrosis (reviewed in (289)).

In addition to promoting proliferation, dissociation of spectrin from the plasma membrane has been connected to other mechanisms of neoplasia. E-cadherin is a critical component of cell-cell contacts, and mislocalization or down-regulation of E-cadherin is often associated with epithelial to mesenchymal transition (EMT) and metastasis (reviewed in (290)). Proper localization of both βIIΣ1 and βIIΣ2-spectrin has been demonstrated to be necessary for proper E-cadherin localization (228, 229, 291). Autocrine GH has recently been shown to induce relocalization of E-cadherin from adherens junctions into the cytoplasm resulting in EMT in MCF-7 cells (292). Based on

these combined findings, it is reasonable to hypothesize that GH-induced, JAK2-mediated phosphorylation of βII-spectrin leads to mis-localization of E-cadherin, contributing to the autocrine GH-induced EMT phenotype in MCF-7 cells. It is interesting to note that I have also found SH2B1β to be localized to adherens junctions in MCF-7 cells (data not shown), raising the possibility that the βII-spectrin/SH2B1β interaction plays a role in this potential mechanism.

Overall, the results presented in Chapter 3 establish the basis for additional studies investigating the functional significance of the SH2B1β-dependent, JAK2-mediated tyrosyl phosphorylation of βII-spectrin and the GH-induced formation of a βII-spectrin/SH2B1/JAK2 complex, as well as the physiological relevance of the GH-induced shift in βII-spectrin localization.

Future Directions

As mentioned above, my inability to co-immunoprecipitate full length βIIΣ1- and βII2-spectrin with SH2B1β may have been due to insufficient solublization of plasma-membrane associated βII-spectrin. Therefore, I will repeat these co-immunoprecipitation experiments with SH2B1β and full-length βIIΣ1- and βIIΣ2-spectrin using 1% TritonX-100 as opposed to 0.1% TritonX-100. Based on the co-immunoprecipitation results in 3T3-F442A cells using the 1% TritonX-100 lysis buffer, I predict that both full-length βII-spectrin isoforms will co-immunoprecipitate with SH2B1β.

One model that can be constructed based on my results in Chapter 3 is that a small fraction of SH2B1 and βII-spectrin exist in a complex in the cytoskeleton prior to GH treatment. Upon GH stimulation, βII-spectrin is recruited with SH2B1β to active JAK2. Active JAK2 phosphorylates βII-spectrin, creating additional binding sites for SH2B1β, and thus increasing the interaction between βII-spectrin and SH2B1β. JAK2-mediated phosphorylation of βII-spectrin additionally results in the translocation of βII-

spectrin out of the plasma membrane and into the cytosol. From the studies by other groups described above that assess the functional consequences of altering spectrin localization, the functional consequences of this model may include GH-induced proliferation, migration or subcellular redistribution of E-cadherin.

I would like to first determine the mechanism of GH-induced formation of the βII-spectrin/SH2B1β/JAK2 complex. Previous studies showed that GH stimulation increases the interaction between SH2B1β and JAK2 (100). My results showed that SH2B1β knockdown decreases the GH-induced interaction between βII-spectrin and JAK2. Taken together, these results suggest that GH may induce SH2B1β to recruit βII-spectrin to JAK2. Figure 3.7 shows that βII-spectrin resides almost completely in the cytoskeletal fraction. In contrast, SH2B1β mostly resides in the soluble fraction in this experiment, while a small portion resides in the cytoskeletal fraction (data not shown). It is important to note that the membrane fraction is included in the soluble fraction in these experiments, as previous reports have shown the majority of SH2B1β to reside in the membrane (132). To determine if GH stimulation induces βII-spectrin redistribution out of the cytoskeletal fraction and into the membrane fraction (where SH2B1β and JAK2 are present), parallel cytoskeleton/non-cytoskeleton and membrane/cytosol fractionation experiments will be performed in 3T3-F442A and H4IIE cells stimulated with GH from 0-60 min. In Fig. 3.8, I showed that GH induced redistribution of βIIΣ2-spectrin out of cell-cell contacts and into the cytoplasm starting at 15 min, with almost complete βIIΣ2-spectrin cytoplasmic localization by 30 minutes. Therefore I predict that GH administration will recruit βII-spectrin out of the cytoskeletal fraction and into the plasma membrane fraction by 15 min following GH stimulation. By 30 min following GH stimulation, I expect the majority of βII-spectrin to be present in the cytoplasm.

To determine the importance of SH2B1β to this proposed subcellular redistribution of βII-spectrin, I will also perform these experiments in SH2B1β knockdown 3T3-F442A cells (shSH2B1β 3T3-F442A cells). My results in Fig 3.7 showed reduced complex formation between βII-spectrin, SH2B1β and JAK2 in shSH2B1β cells compared to shControl cells. Therefore, I predict that GH will induce less βII-spectrin to be redistributed into membrane and cytoplasmic fractions in shSH2B1β cells compared to shControl cells.

Finally, the model proposed above predicts that JAK2-mediated phosphorylation of βII-spectrin is necessary for GH to induce βII-spectrin to redistribute into the cytoplasm. To test this, tyrosine phosphorylation of βII-spectrin will also be monitored in the different subcellular fractions over the GH time course. Additionally, if the tyrosine(s) phosphorylated by JAK2 are identified (see below), I will express βII-spectrin tyrosine to phenylalanine mutants in this system. I expect that GH stimulation will not elicit redistribution of βII-spectrin tyrosine to phenylalanine mutants into the cytoplasm.

To identify the tyrosine(s) phosphorylated by JAK2, I will create βIIΣ1- or βIIΣ2-spectrin truncation mutants. These mutants will be expressed in 293T cells with SH2B1β and JAK2 to identify the region of βII-spectrin that is tyrosyl phosphorylated. Following identification of the region of βII-spectrin that is tyrosyl-phosphorylated by JAK2, I will create tyrosine to phenylalanine point mutants to identify the individual tyrosine(s) on βII-spectrin that are phosphorylated by JAK2. Assuming positive results are achieved in these experiments, *in vitro* kinase assays will be employed to determine if JAK2 directly phosphorylates βII-spectrin on tyrosines. The model predicts that tyrosyl phosphorylation of βII-spectrin creates additional binding sites for SH2B1β. Therefore, these tyrosine to phenylalanine point mutants will be utilized in co-immunoprecipitation

assays with SH2B1β. I predict that less SH2B1β will co-immunoprecipitate with mutant βII-spectrin compared to wild type βII-spectrin.

I would like to characterize the functional consequence of GH-induced βII-spectrin-containing complexes, potential βII-spectrin tyrosyl phosphorylation and βII-spectrin subcellular re-distribution. I will create and express SH2B1β truncation mutants lacking the βII-spectrin binding domain, or βII-spectrin mutants lacking the SH2B1β binding domain or lacking tyrosines phosphorylated by JAK2 in 3T3-F442A, H4IIE or autocrine GH-expressing MCF-7 cells. If βII-spectrin phosphorylation or subcellular redistribution is necessary for GH-induced proliferation or migration, I predict that expression of these mutants will act as dominant negative proteins in assays of GH-induced proliferation and/or migration.

Identification and characterization of SH2B1β as a novel focal adhesion protein

In Chapter 4, I further solidify the role of SH2B1β as a component and/or regulator of the actin cytoskeleton by the identification of SH2B1β as a focal adhesion protein. Fluorescently tagged SH2B1β consistently localized to focal adhesions in 3T3-F442A and NIH 3T3 fibroblasts as well as MCF-7 and HeLa epithelial-derived cells, indicating that SH2B1β is a component of focal adhesions in multiple cell types. Focal adhesions are integrin-based macromolecular complexes that mediate cell-extracellular matrix (ECM) attachment and allow direct signaling between the extracellular matrix and the cell (reviewed in (128)).

The finding that the SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization leads to the hypothesis that SH2B1β binds a tyrosyl phosphorylated protein within the focal adhesion complex. Focal adhesions are known to contain multiple tyrosyl-phosphorylated proteins (including paxillin (267), vinculin (268,

269), talin (270) p130Cas (271), tensin (272), and FAK (267)). Additionally, the SH2 domains of other focal adhesion proteins have been identified as important for focal adhesion localization (273, 274) and overall tyrosine phosphorylation of focal adhesion proteins is thought to be an important regulatory mechanism of focal adhesion formation, stability and maturation (reviewed in (275)). Therefore, it is possible that mechanisms regulating overall focal adhesion protein tyrosyl phosphorylation (eg. Src and FAK activation) also regulate the recruitment of SH2B1β to focal adhesions. However, I have eliminated both FAK and paxillin as potential SH2B1β interacting molecules. We have identified two additional focal adhesion proteins, vinculin and talin, whose sites of tyrosyl phosphorylation make them attractive candidates for SH2B1β binding proteins. SH2B1β is known to bind phosphotyrosines that lie within a YXXL motif (12). Vinculin tyrosine 822 and talin tyrosines 270 and 2530 are all reported to be phosphorylated and all reside within a YXXL motif (269, 293). Future experiments will explore the possibility that SH2B1β interacts with one or both of these proteins within the context of tyrosine phosphorylation.

All SH2 domain-containing SH2B1β mutants were able to localize to focal adhesions. GFP-SH2B1β (118-670), lacking the dimerization domain, GFP-SH2B1β (Δ150-200), lacking the NLS, and GFP-SH2B1β mNES all displayed focal adhesion localization similar to WT. However, GFP-SH2B1β (269-670), which contains an intact SH2 domain but lacks the dimerization domain, NLS and NES, appeared to display reduced focal adhesion localization compared to WT GFP-SH2B1β (Fig. 4.3). These data indicate that while individual disruption of the dimerization domain, NLS or NES by deletion or point mutation does not affect focal adhesion localization, collective disruption of these regions does have an effect. Alternatively, deleting amino acids 1-

268 may produce a conformational change in the remaining SH2B1β protein that results in atypical protein localization.

In addition to SH2B1β, I found SH2B1γ and SH2B1δ to localize to focal adhesions. I predict that localization of these SH2B1 isoforms is also mediated through the SH2 domain of SH2B1, and I therefore expect that SH2B1α will also localize to focal adhesions through its SH2 domain when tested in future experiments. SH2B2 and SH2B3 both contain SH2 domains similar to SH2B1, and are known to bind at least some of the same partners as SH2B1 (22, 276, 294). Therefore it is possible that the other SH2B family members are also focal adhesion proteins. Interestingly SH2B3 has been described as a binding partner of the focal adhesion protein, filamin (139). However, this interaction was not mediated through the SH2 domain of SH2B3. Therefore, it is possible that different SH2B family members are targeted to focal adhesions through differing mechanisms.

There are currently greater than 150 proteins identified as focal adhesion proteins and almost 700 identified interactions between focal adhesion proteins, making these structures among the most complex assembly of proteins within a cell (see (275) and (128) for reviews}. Although the term “focal adhesion” is generally used to denote these structures, at least three distinct cell-ECM adhesion complexes have been defined in motile cells based on their temporal formation and protein composition (reviewed in (295)). Focal complexes are the earliest integrin-based adhesions to form, can be seen underneath the leading edge of lamellipodia, are highly vinculin and paxillin positive and contain a high concentration of tyrosyl-phosphorylated proteins (296-298). These focal complexes are the initial sites of attachment at the leading edge of lamellipodia. They have been hypothesized to tether the actin meshwork at the leading edge of lamellipodia to a solid substrate, and thus act to direct the force of actin polymerization towards the

leading edge. Some focal complexes mature into focal adhesions, which are located at the edges of the cell and dispersed underneath the body of the cell. Like focal complexes, focal adhesions are highly vinculin and paxillin positive and contain many tyrosyl-phosphorylated proteins. However, unlike focal complexes, focal adhesions are also zyxin and tensin positive and incorporate actin bundles into the adhesion site (297). Focal adhesions can further mature into fibrillar adhesions, which are typically located more centrally within the cell and are elongated, rather than punctate adhesion sites. While fibrillar adhesions are composed largely of the same proteins that make up focal adhesions, fibrillar adhesions display a higher concentration of tensin than focal adhesions and contain little or no tyrosyl-phosphorylated proteins (298). Both focal adhesions and fibrillar adhesions are thought to provide traction, monitor mechanical forces and monitor the composition of the ECM to facilitate and regulate cell motility. Therefore, while motile cells contain three major types of adhesions sites that perform varying functions, there are currently only a few known differences in protein composition between focal complexes, focal adhesions and fibrillar adhesions.

Interestingly, I found SH2B1β to constitutively localize to both focal adhesions and fibrillar adhesions, but I was unable to conclusively find SH2B1β present in vinculin positive focal complexes (Figure 4.1B, bottom left image, punctate dots are focal adhesions and slashes are fibrillar adhesions; data not shown). This raises the possibility that, like zyxin and tensin, SH2B1β is specifically a component of more mature adhesion sites. While the functional consequences of zyxin and tensin incorporation into mature adhesion sites is unknown, it has been hypothesized that these proteins add structural stability to adhesion sites (297). Focal adhesions have half-lives of 10-30 min while focal complexes have half-lives of only a few minutes (reviewed in (295)). The turnover of these adhesion complexes may be related to the

dynamic cycling of focal adhesion proteins into and out of focal adhesions themselves (called focal adhesion protein turnover dynamics) (252). Therefore, proteins that exhibit reduced turnover dynamics or reduce the turnover dynamics of other focal adhesion proteins may in turn reduce the overall turnover of cell adhesion sites. It is possible that SH2B1β functions in a similar fashion to add stability to these adhesion sites. The dynamic turnover of vinculin and paxillin are often monitored to assess the overall turnover rates of focal adhesions (129). I found that co-expression of SH2B1β with vinculin does not alter vinculin focal adhesion turnover dynamics compared to vinculin expressed alone (data not shown), possibly excluding this hypothesis. Alternative potential functions of SH2B1β in focal adhesions are discussed in greater detail below.

The data presented in Chapter 4 show that SH2B1β is present in focal adhesions prior to stimulation by GH or other stimuli such as serum. However, GH stimulation increases the dynamic cycling of SH2B1β into and out of focal adhesions (SH2B1β focal adhesion turnover) (Fig. 4.4). In additional experiments, I found that SH2B1β focal adhesion turnover was increased in cells cultured in serum-containing medium compared to cells cultured in serum-free medium. These observations raise the question of how GH and other stimuli act on focal adhesion-localized SH2B1β. Although several growth factor receptors have been reported to localize to focal adhesions, GHR has not been reported in focal adhesions and in additional experiments, I was unable to detect GFP-GHR in focal adhesions. This seems to rule out the possibility that SH2B1β associates with and is regulated by a GHR/JAK2 complex at focal adhesions. It is possible that GH and other stimuli induce JAK2 or other regulators to translocate to focal adhesions to modify SH2B1β, thus altering its focal adhesion turnover dynamics. However, I did not observe JAK2 to localize to focal adhesions under serum-deprived, GH-stimulated or serum-stimulated conditions. Alternatively, GH or other stimuli modify

SH2B1β at the plasma membrane (e.g. JAK2 phosphorylates SH2B1β at the activated GHR/JAK2 complex). This modified SH2B1β may then be incorporated into focal adhesions through the normal process of focal adhesion protein turnover. As the concentration of modified SH2B1β in the cell increases, the concentration of modified SH2B1β within focal adhesions will also increase, thus altering SH2B1β turnover dynamics at focal adhesions. Future experiments described below will test these hypotheses.

The fact that rates at which individual proteins cycle into and out of focal adhesions can directly affect the rates of focal adhesion turnover (129, 252) and cell motility raises the possibility that one mechanism whereby GH induces cell motility is through altering the focal adhesion turnover dynamics of SH2B1β. Therefore, it will be of interest to determine whether inhibiting the ability of GH to alter SH2B1β turnover dynamics also inhibits GH-induced cell motility. GH stimulation induces phosphorylation of SH2B1β at tyrosines 439 and 494, and phosphorylation of these tyrosines is thought to be required for SH2B1β enhancement of GH-induced cell motility (116). I found that mutation of both tyrosines 439 and 494 in combination does not affect basal focal adhesion turnover rates of SH2B1β in 3T3-F442A cells cultured in the presence of serum (data not shown). However, to assess the effect of phosphorylation of tyrosines 439 and 494 on the ability of GH to increase the turnover rate of SH2B1β at focal adhesions, these experiments need to be repeated in cells treated with GH following a period of serum deprivation.

Although GH stimulation is primarily thought to result in tyrosyl phosphorylation of SH2B1β, NGF and PMA stimulation is thought to result in SH2B1β becoming highly serine/threonine phosphorylated (224). In Fig. 4.5, I show that PKC mediates a PMA-induced upward shift in endogenous SH2B1 mobility, as assessed by Western blot. This

upward shift is consistent with SH2B1β being serine/threonine phosphorylated since it is reversed by incubation with a phosphatase (224). Recent work in the Carter-Su laboratory has identified at least two serines, serine 161 and serine 165, as playing an important role in the regulation of SH2B1β at the plasma membrane in response to PMA in neuronal PC12 cells (Maures, et al. Manuscript in preparation). Mass spectroscopy identified serine 161 as being phosphorylated in cells stimulated with PMA. Whether serine 165 is also phosphorylated is unknown. Both of these serines lie within classic PKC substrate phosphorylation motifs [X(S/T)X(R/K)], and therefore, may be phosphorylated by PKC. I found that PMA stimulation resulted in less of an upward mobility shift of GFP-SH2B1β (S161,165A) than WT GFP-SH2B1β in 3T3-F442A cells. These results suggest the possibility that PMA stimulates a PKC-mediated phosphorylation of SH2B1β at serines 161 and/or 165 in 3T3-F442A cells.

In Chapter 4, I also show that PMA stimulation causes WT SH2B1β, but not SH2B1β (S161,165A), to leave focal adhesions. PKC α, δ and ε are found in focal adhesions (254-256) and PKC is thought to directly phosphorylate vinculin (257), talin (258), filamin (259) and integrin (277) at focal adhesions. Taking these results into account, I hypothesize that PMA induces PKC to phosphorylate SH2B1β at serines 161 and/or 165, which causes SH2B1β to leave focal adhesions. Adding PMA in the presence of PKC-specific inhibitors and examining whether PMA causes SH1B1β to leave focal adhesions would lend additional support for PKC in this mechanism.

Serines 161 and 165 lie within a 30 amino acid stretch of SH2B1β that contains 13 serines and threonines. Ten of these serines and threonines (S137, S141, T142, T143, S144, S145, S154, S157, S161, S165) lie within the classic PKC-substrate motif, X(S/T)X(R/K) (Center for Biological Sequence Analysis, http://www.cbs.dtu.dk/index.shtml). Of these many potential PKC phosphorylation sites,

serines 161 and 165 appear particularly important for SH2B1β movement out of focal adhesions due to several additional experiments that I have recently performed. First, experiments with SH2B1β containing mutation of 11 additional serines and threonines in combination with serines 161 and 165 [SH2B1β (13SA)] gave the same results as SH2B1β (S161,165A). Second, when these experiments were performed on SH2B1β (13SA) with alanine 165 mutated back to serine [SH2B1β (13SA,A165S)], the results were almost identical to WT SH2B1β. This raised the possibility that serine 165 alone was responsible for the PMA-induced redistribution out of focal adhesions. However, when these experiments were performed with SH2B1β (S165A), the results were again almost identical to WT SH2B1β. Together, these results suggest that PMA-induced phosphorylation of either serine 161 or 165 is sufficient for SH2B1β to leave focal adhesions. This comes from the fact that WT SH2B1β, SH2B1β (13SA,A165S), and SH2B1β (S165A) all have the same phenotype (ie. PMA induces their dissociation from focal adhesions), and that SH2B1β (S161,165A) and SH2B1β (13SA) have the same phenotype (ie. PMA does not induce their dissociation from focal adhesions). I predict that future experiments using SH2B1β (S161A) will also result in a phenotype similar to that of WT SH2B1β.

In the PC-12 model, it is thought that the negatively charged plasma membrane recruits SH2B1β through its positively charged amino acids within the NLS. Phosphorylation of Ser 161/165 (which lie adjacent to the positively charged NLS) would add a negative charge to the vicinity of the NLS, counteracting its positive charge, and acting as an electrostatic switch. This is thought to induce dissociation of SH2B1β from the PM. In Fig. 4.3, I show that the NLS is dispensable for SH2B1β focal adhesion localization. This indicates that it is not necessary for SH2B1 to be localized to the plasma membrane in order to localize to focal adhesions. It also indicates that the

proposed phosphorylation of Ser 161/165 causes SH2B1β dissociation from focal adhesions by some mechanism other than that of an electrostatic switch. For example, a phosphorylation-induced conformational change could lead to a decreased affinity of SH2B1β for some binding partner. Serine phosphorylation of vinculin (283) and paxillin (284) induces dissociation of these proteins from focal adhesions, although the mechanism of dissociation is also unknown. Therefore, future investigation into the precise mechanism of PMA-induced SH2B1β release from focal adhesions is warranted, and may shed light on a general mechanism whereby serine phosphorylation regulates localization of focal adhesion proteins.

The results suggesting a phosphorylation-induced release of SH2B1β from focal adhesions are supported by the SH2B1β turnover experiments in Chapter 4 that show that compared to WT SH2B1β, SH2B1β (S161,165A) has reduced turnover dynamics and SH2B1β (S165E) has increased turnover dynamics. I found that SH2B1β (13SA) displayed turnover dynamics almost identical to SH2B1β (S161,165A) in both serum-deprived and serum-fed conditions. I predict the turnover dynamics of SH2B1β (S161E) will be found to be similar to those of SH2B1β (S165E). These experiments suggest that phosphorylation of SH2B1β at serine 161 and/or 165 regulates the dynamic turnover of SH2B1β at focal adhesions. Serine phosphorylation of paxillin has been shown to regulate the dynamic turnover of paxillin at focal adhesions (129), indicating that phosphorylation of focal adhesion proteins may be a general mechanism regulating focal adhesion protein turnover.

These differences in SH2B1β turnover dynamics due to mutation of serines 161/165 are likely to be related to the PMA-induced dissociation of SH2B1β from focal adhesions. PMA stimulation induces strong activation of several PKC isoforms. Cells are not normally exposed to conditions such as this, but rather are exposed to lower

levels of PKC activation in response to various cytokines, growth factors and other external and internal stimuli. Therefore, rather than the all or nothing localization response observed in the PMA stimulation experiments, a cell will more likely be exposed to conditions that either increase or decrease the dynamic cycling SH2B1β into and out of focal adhesions. I hypothesize that conditions that induce the proposed phosphorylation of serines 161 and 165 will push the balance of SH2B1β focal adhesion dynamics towards increased cycling into and out of focal adhesions (i.e. less SH2B1β in focal adhesions and/or SH2B1β remaining in focal adhesions for a shorter period of time). Conversely, I hypothesize that conditions that promote dephosphorylation of serines 161 and 165 will push the balance of SH2B1β focal adhesion dynamics towards decreased cycling into focal adhesions (i.e. more SH2B1β in focal adhesions for a longer period of time).

The functional consequences of changes in SH2B1β focal adhesion turnover dynamics seem to support this hypothesis. Although the data were not quantified in Chapter 4, it appeared that focal adhesions in cells expressing GFP-SH2B1β (S161,165A) were brighter (more intense fluorescence) than focal adhesions in cells expressing WT SH2B1β. Conversely, focal adhesions in cells expressing GFP-SH2B1β (S165E) appeared dimmer (less intense fluorescence) than focal adhesions in cells expressing WT GFP-SH2B1β. As described above, a slower SH2B1β (S161,165A) turnover rate would lead to more SH2B1β in focal adhesions and vice versa for SH2B1β (S165E). The dynamic turnover of SH2B1β at focal adhesions also appeared to affect focal adhesion size, as cells expressing GFP-SH2B1β and GFP appeared to have focal adhesions of the same size, whereas focal adhesions in cells expressing GFP-SH2B1β (S161,165A) were larger and those in cells expressing GFP-SH2B1β (S165E) were smaller. A finding that these differences in focal adhesion size are statistically significant

would implicate the regulation of SH2B1β focal adhesion turnover dynamics as an important mediator of overall focal adhesion characteristics.

The final piece of data presented in Chapter 4 revealed the surprising fact that expression of SH2B1β (S165E) alone resulted in a significant increase in the overall number of focal adhesions per cell. Increased dynamic cycling of SH2B1β (S165E) appears necessary for this phenotype as opposed to decreased levels of SH2B1β (S165E) in focal adhesions. This conclusion is based on the observation that SH2B1β truncation mutants that do not localize to focal adhesions [e.g. SH2B1β (1-555), SH2B1β (1-260)] or that appear to exhibit reduced focal adhesion localization [e.g. SH2B1β (269-670) do not increase the overall number of focal adhesions per cell. Interestingly, PKC activation is able to recruit activated ERK1/2 to focal adhesions (253) as well as activate Rho-A (280), events which lead to an increase in the focal adhesion number. It is tempting to hypothesize that PKC-mediated phosphorylation of SH2B1β at serine 161 and/or 165 plays a role in PKC-mediated recruitment of ERK1/2 to focal adhesions and/or activation of RhoA.

As discussed above, the functional consequences of the differences between composition of focal complexes and more mature adhesions sites have not been elucidated. However, the signals that initiate the formation of focal complexes and focal adhesions have been characterized. Rac-1 activity is well known to initiate focal complex formation (reviewed in (299)) while focal adhesion formation is dependent on Rac-1 inactivation and Rho-A activation (300). In relation to these mechanisms of adhesion formation, it is worth noting that SH2B1β has been found to constitutively bind Rac (112). However, I predict that focal adhesion-localized SH2B1β does not bind Rac, as this would inhibit the actual formation of focal adhesions. Because SH2B1β is already known to bind one small GTPase (Rac-1), it may be worthwhile for future studies

to investigate the possibility that SH2B1β interacts with additional small GTPases such as Rho-A. Alternatively, SH2B1β may recruit GTPase regulator or effector molecules to focal adhesions. In unpublished data, the Carter-Su lab has identified Vav1 and Vav2 as SH2B1β binding partners. Vav proteins are guanine nucleotide exchange factors (GEFs) for small GTPases (301-304). Vav2 has been shown to localize to focal adhesions (305), and expression of constitutively active Vav has been shown to induce focal adhesion formation in a manner similar to constitutively active Rho-A (306). Therefore, it is possible that SH2B1β regulates focal adhesion dynamics by recruiting Vav proteins to focal adhesions. In support of this hypothesis, in preliminary experiments, I found GH stimulation of 3T3-F442A cells expressing GFP-Vav2 to result in the recruitment of GFP-Vav2 into the region of focal adhesions, but only when mCherry-SH2B1β was co-expressed. Additionally, focal adhesions in GH-stimulated cells co-expressing Vav2 and SH2B1β appeared smaller and finer than in unstimulated cells or cells expressing SH2B1β alone. Khosravi-Far et. al reported a similar phenotype when they expressed constitutively active Vav or Rho-A in NIH 3T3 cells (306).

Taking these observations together, I hypothesize that stimuli that induce phosphorylation of SH2B1β on serines 161 and/or 165 increase the rate of SH2B1β turnover dynamics at focal adhesions. This, in turn, could allow for increased Vav targeting to focal adhesions as SH2B1β quickly cycles into and out of focal adhesions. Alternatively, phosphorylation of SH2B1β at serines 161/165 may increase the affinity of Vav for SH2B1β. In either case, the interaction between SH2B1β and Vav may result in Rho-A activation which would be predicted to promote the formation of new focal adhesions. In support of this hypothesis, as described above, cells expressing GFP-SH2B1β (S165E) (mimicking phosphorylation of S165) alone exhibit smaller and more focal adhesions that appear strikingly similar to focal adhesions in GH-treated cells

expressing both Vav2 and SH2B1β. Clearly, additional experiments are needed to establish that PKC mediates phosphorylation of SH2B1β at serines 161/165 and elucidate the mechanism whereby that modification alters SH2B1β focal adhesion turnover dynamics and increases overall focal adhesion number.

Currently, no fractionation protocols exist to allow complete isolation of focal adhesions proteins, and therefore, it is impossible to determine with complete confidence whether PKC directly phosphorylates SH2B1β in focal adhesions. However, *in vitro* kinase assays assessing direct phosphorylation of SH2B1β by specific PKC isoforms, PKC-specific inhibitor assays testing the necessity of PKC to regulate SH2B1β at focal adhesions, and RNAi experiments evaluating the effect of reducing specific PKC isoforms can all be employed in future studies to more completely investigate the role of PKC in regulating SH2B1β at focal adhesions.

One final additional mechanism by which SH2B1β may be involved in focal adhesion function relates to the emerging role of focal adhesion proteins in the nucleus. Intriguingly, proteins (e.g. Vav, VASP, paxillin and the zyxin family of proteins (reviewed in (307)) have recently been observed to shuttle between focal adhesion complexes and the nucleus. The mechanism of nuclear translocation for most of these proteins is unknown, as most do not contain an NLS. The Carter-Su lab has shown that SH2B1β contains both an NLS and NES, undergoes nucleocytoplasmic shuttling (106, 107) and binds at least one focal adhesion protein (Vav). In preliminary experiments, I observed accumulation of SH2B1β in the nucleus of 3T3-F442A cells when co-expressed with Vav2. It is possible, therefore, that SH2B1β translocates to the nucleus with, or alters the nuclear dynamics of, one or more focal adhesion proteins. Future experiments utilizing photoactivatable-tagged SH2B1β can assess the ability of stimuli such as GH or PMA to induce SH2B1β shuttling between focal adhesions and the nucleus. In addition,

blocking SH2B1β nuclear import may reveal SH2B1β as a shuttling protein for one or more focal adhesion proteins.

Future Experiments

While I have demonstrated that the SH2 domain of SH2B1β is necessary and sufficient for focal adhesion localization, I have not yet determined what the SH2 domain binds within focal adhesions. As mentioned above, the focal adhesion proteins talin and vinculin, are both phosphorylated on tyrosines lying within YXXL motifs. Because SH2B1β is known to bind pYXXL, I plan to perform co-immunoprecipitation experiments with SH2B1β and talin or vinculin. I have obtained cDNA encoding these proteins and have performed preliminary experiments. Unfortunately, I have run into several technical difficulties in these preliminary experiments. Both talin and vinculin are GFP-tagged, and I have currently co-expressed GFP-vinculin with Flag-tagged SH2B1β in HeLa and 293T cells. In each case, our anti-Flag conjugated agarose beads efficiently pulled down Flag-SH2B1β as well as GFP-vinculin. However, GFP alone was also pulled down, indicating that the anti-Flag beads non-specifically bind GFP. I subsequently expressed GFP-vinculin or GFP-talin with myc-SH2B1β in 293T cells and immunoprecipitated with anti-myc antibody. In this experiment, GFP-vinculin co-immunoprecipitated with myc-SH2B1β, but also with the empty myc-vector control, again indicating non-specific co-immunoprecipitation. Although it appeared that a small amount of GFP-talin might have co-immunoprecipitated with myc-SH2B1β, the band was too faint to draw any conclusions. I subsequently found by confocal microscopy that 293T cells do not appear to have any distinguishable focal adhesions, making this cell line a poor choice for detecting interactions between focal adhesion proteins. In future experiments, I plan to express GFP-talin or GFP-vinculin in 3T3-F442A cells with myc-SH2B1β and perform parallel myc and GFP immunoprecipitations. Michael Doche, a

fellow graduate student in the Carter-Su laboratory, is performing a tandem affinity purification (TAP)-tagged SH2B1β immunoprecipitation assay using 3T3-F442A cells. It is possible that his experiment will also yield an SH2B1β-interacting focal adhesion protein.

I am also interested in whether other SH2B family members are focal adhesion proteins, and therefore plan to express GFP-tagged SH2B2 and SH2B3 in 3T3-F442A cells and visualize them by confocal microscopy. Because of their shared SH2 domains and shared ability of their SH2 domains to bind phosphorylated tyrosine 813 in JAK2, I expect that both proteins will localize to focal adhesions. Also, because SH2B3 has been shown to interact with the focal adhesion protein, filamin, it would be interesting to perform co-immunoprecipitation experiments with each SH2B family member and filamin.

In Fig. 4.4, I showed that GH stimulation increases SH2B1β focal adhesion turnover. I would like to determine if this effect is due to GH-induced tyrosyl phosphorylation of SH2B1β. Therefore, I plan to perform additional FRAP experiments on focal adhesions in 3T3-F442A cells expressing WT GFP-SH2B1β or GFP-SH2B1β (Y439,494F) in serum-deprived and GH-stimulated conditions. If GH-induced tyrosyl phosphorylation of SH2B1β is required for GH-induced increases in SH2B1β focal adhesion turnover rates, I predict that GH stimulation will be unable to increase GFP-SH2B1β (Y439,Y494) focal adhesion turnover rates. A positive finding would suggest that the SH2B1β in focal adhesions after GH stimulation has been phosphorylated by JAK2. This raises the question of whether JAK2 phosphorylates SH2B1β at focal adhesions or whether SH2B1β that was associated with the GHR/JAK2 complex at the plasma membrane translocates to focal adhesions. To address this question, SH2B1β could be tagged with a photoactivatable tag. Immediately prior to, or during GH

stimulation, the tag of either focal adhesion-localized or plasma membrane localized SH2B1β could be photoactivated and then traced over a period of time. Because I have not been able to identify JAK2 in focal adhesions, I expect that SH2B1β that is associated with JAK2 at the plasma membrane will translocate to focal adhesions, thus accounting for the increased SH2B1β focal adhesion turnover observed upon GH stimulation.

I believe that establishing the functional significance of SH2B1β in focal adhesions is among the most important issues remaining for this project. In initial experiments, I found 3T3-F442A cells stably overexpressing GFP-SH2B1β to spread significantly faster than cells stably expressing GFP alone. Conversely, I found SH2B1 knockout MEFs to spread significantly slower than their WT MEF counterparts. These data suggest that levels of SH2B1β in focal adhesions have a general effect on focal adhesion function (cell spreading assays are commonly used to assess the effect of increasing or decreasing focal adhesion protein levels). I attempted to repeat these experiments with 3T3-F442A cells transiently expressing GFP-SH2B1β (S161,165A) or GFP-SH2B1β (S165E). However, I observed no differences between these cells and cells transiently expressing WT GFP-SH2B1β or GFP alone. It is possible that stable cell lines are needed for this assay to succeed.

To more directly assess the contribution of focal adhesion localized SH2B1β to GH-induced responses, I will test the effect of WT, (S161,165A) and (S165E) SH2B1β on GH-induced cell migration. In unpublished data, Dr. Hsiao-Wen Su, a postdoctoral fellow in the Carter-Su laboratory, has recently shown WT SH2B1β to enhance GH-induced cell motility in RAW 264.7 macrophages. In addition, she found that SH2B1β (S161,165A) inhibits GH-induced motility while SH2B1β (S165E) enhances basal motility to levels above that of GH inducement in these same cells. These results are consistent

with what I would predict based on my characterization of SH2B1β as a focal adhesion protein. However, focal adhesions are difficult to visualize in RAW 264.7 macrophages, and therefore, characterization of SH2B1β focal adhesion dynamics in these cells may not be possible. I have attempted to repeat these experiments in 3T3-F442A cells using transwell migration assays. However, I have been unable to observe GH-induced motility in this model. In fact, using live cell confocal microscopy, I failed to detect any motility in 3T3-F442A cells in response to GH, EGF or FGF stimulation over a period of 1 hour. This indicates that 3T3-F442A cells are not the optimal model to assess effects of SH2B1β on cell motility. However, while performing the live cell confocal microscopy experiments, I observed GH, EGF and FGF to induce lamellipodia extension and retraction as well as changes in overall cell morphology. Formation and dissolution of focal adhesions accompanied these processes. Cells expressing GFP, WT GFP-SH2B1β and GFP-SH2B1β (S165E) appeared to all respond similarly. However, cells expressing GFP-SH2B1β (S161,165A) appeared to be unable to extend lamellipodia or change their morphology as fluidly as the other cells. In fact, I commonly observed GFP-SH2B1β (S161,165A) expressing cells attempting to retract a cellular extension, and in the process, tear themselves apart. Interestingly, focal adhesions remained at the ends of where the extension had been before the cells were torn. This raises the possibility that these cells were unable to properly dissolve focal adhesions, a function that is necessary for cellular motility. I would like to repeat the transwell assays in the MCF-7 model that stably expressed GH. These cells are known have increased motility compared to control MCF-7 cells (308). I predict that results in these cells will be similar to those in the RAW 264.7 macrophage model in part based on some results that I did observe in 3T3-F442A cells.

Summary

The overall goal of my thesis research was to answer some of the important remaining questions in GH signal transduction. In particular, I wanted to identify the primary kinase(s) responsible for transducing the GH signal in 3T3-F442A and H4IIE cells and then more fully characterize the role of the adaptor protein, SH2B1β, in GH-induced regulation of the actin cytoskeleton. I have shown that JAK2 is primarily responsible for GH-induced activation of Stat1, Stat3, Stat5, ERK1/2 and Akt in both 3T3-F442A and H4IIE cells. I have additionally shown that a major component of the actin cytoskeleton, βIIΣ1-spectrin, is a novel SH2B1β binding partner that is phosphorylated by JAK2 in an SH2B1β-dependent manner. I have further demonstrated that GH stimulation of 3T3-F442A cells induces the formation of a βII-spectrin/SH2B1β/JAK2 complex, and show that GH stimulation of H4IIE cells results in a redistribution of βIIΣ2-spectrin from cell-cell contacts to the cytoplasm. Finally, I have identified SH2B1β as a novel focal adhesion protein whose focal adhesion turnover dynamics are regulated by GH stimulation in 3T3-F442A cells. I have also demonstrated that PMA stimulation of 3T3-F442A cells induces a rapid redistribution of SH2B1β out of focal adhesions. I have provided evidence suggesting that phosphorylation of two serines within SH2B1β regulates both this PMA-mediated event, SH2B1β turnover at focal adhesions, and overall focal adhesion number. SH2B1β is an adaptor protein in several signal transduction pathways, and therefore we predict that SH2B1β localization to focal adhesions and the SH2B1β/βII-spectrin interaction are relevant to many other ligands. In addition, because SH2B1β localizes to focal adhesions in the absence of any ligand stimulation, we predict that SH2B1β regulates focal adhesion function in general.

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