A Dissertation

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Prolactin-Dependent Regulation of the Actin Cytoskeleton by JAK2, SH2B1β, PAK1 and Filamin A

by

Leah Rider

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biology

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The University of Toledo
August 2011
An Abstract of

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JAK2 is a receptor-associated tyrosine kinase responsible for signaling involving most of the cytokine family of receptors, including the prolactin (PRL) receptor (Murata, Noguchi et al. 1995; Argetsinger and Carter-Su 1996; Roy and Cathcart 1998; Parham, Chirica et al. 2002). p21-activated kinase (PAK1) is a serine-threonine kinase which participates in many important biological processes, including morphogenesis, cell survival, mitosis, transformation and regulation of the microtubule and actin cytoskeletons (Bokoch 2003; Zhao and Manser 2005; Kumar, Gururaj et al. 2006). Recently we have shown that PAK1 is a novel substrate for JAK2, which directly phosphorylates PAK1 in response to PRL in vivo and in vitro (Rider, Shatrova et al. 2007). SH2B1β is another substrate for JAK2, which binds to and increases the tyrosine kinase activity of JAK2 (Rui, Mathews et al. 1997; Rui and Carter-Su 1999). SH2B1β is an adaptor protein that participates in both cytokine and growth factor signaling and is involved in regulation of the actin cytoskeleton. SH2B1β promotes cytoskeletal rearrangement, inducing membrane ruffling and lamellipodia formation, as well as increasing cellular motility (Herrington, Diakonova et al. 2000; Diakonova, Gunter et al.
We have recently shown that SH2B1β binds directly to F-actin and increases membrane ruffling in response to PRL (Rider, Tao et al. 2009). PAK1 has numerous actin-regulating substrates and we have found that one of these substrates - Filamin A (FLNa), an actin cross-linking protein – functions downstream of pTyr-PAK1. We show that FLNa also associates with SH2B1β in vivo and in vitro. We are interested in the role of both JAK2 substrates – PAK1 and SH2B1β – in the regulation of the actin cytoskeleton and cell motility. We hypothesize that JAK2, PAK1, SH2B1β and FLNa work in complex together in order to mediate cytoskeletal rearrangement in response to PRL.
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List of Abbreviations

BSA................. Bovine serum albumin
CAK.................. CDK-activating kinase
CDK.................. Cyclin-dependent kinase
cDNA............... Complementary deoxyribonucleic acid
CRIB.................. Cdc42/Rac interactive binding
EGF.................. Epidermal growth factor
FBS.................. Fetal bovine serum
FGF.................. Fibroblast growth factor
GST................. Glutathione-S-Transferase
HRP................. Horseradish peroxidase
IB................... Immunoblotting
IGF-1.............. Insulin-like growth factor-1
IP................... Immunoprecipitation
JAK2............... Janus kinase 2
JNK................ c-Jun N-terminal kinase
LSB................ Laemmli sample buffer
MAPK................ Mitogen-activated protein kinase
NGF................ Nerve growth factor
NES................ Nuclear export signal
NLS................ Nuclear localization signal
PAK1............... p21-activated kinase 1
PBD................ p21-binding domain
PBS.....................Phosphate buffered saline
PCR.......................Polymerase chain reaction
PDGF....................Platelet derived growth factor
PEI.......................Polyethyleneimine
PH........................Pleckstrin homology
PI3K.....................Phosphatidylinositol 3 kinase
PIX......................PAK-interacting exchange factor
PMSF....................Phenylmethanesulphonylfluoride
PRL......................Prolactin
PRLR....................Prolactin receptor
PTB......................Phosphotyrosine-binding
pY........................Phosphotyrosine
SDS-PAGE...............Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SH2......................Src homology 2
STAT....................Signal transducer and activator of transcription
TBS......................Tris buffered saline
VEGF....................Vascular endothelial growth factor
WT.......................Wild type
I. Introduction

Prolactin

Prolactin (PRL) is a peptide hormone secreted by the pituitary gland, originally named for its ability to stimulate lactation. PRL is a member of the lactogen family of proteins, including placental lactogen and growth hormone, among others (Teilum, Hoch et al. 2005). Though both placental lactogen and growth hormone can bind the PRL receptor (PRLR), PRL binds only to the PRLR (Ben-Jonathan, LaPensee et al. 2008). PRL participates in over 300 different biological functions, acting via endocrine, autocrine or paracrine mechanisms (Bernichtein, Touraine et al. 2010). PRL is not only secreted by the pituitary, but also the mammary gland, skin, adipocytes, the prostate gland, immune cells and other tissues (Ben-Jonathan, LaPensee et al. 2008). There are seven different isoforms of the human PRLR that have been identified to date, including the most common, the long PRLR isoform (Clevenger, Gadd et al. 2009). PRL binding to its receptor results in the subsequent phosphorylation of the receptor and initiation of multiple signaling pathways, including Grb2-Sos-Ras-Raf-MEK-MAPK (Harris, Stanford et al. 2004), Nek3-Vav2-Rac1 (Miller, DeMaria et al. 2005; Miller, Antico et al. 2007) and perhaps the best-characterized pathway, JAK2-Stats (Clevenger 2004) (Figure 1).

PRL has also been implicated in human breast cancer (Clevenger, Gadd et al. 2009). Greater than 80% of human breast tumors express the PRLR (Touraine, Martini et al. 1998) while PRL production is upregulated in many breast carcinomas (McHale, Tomaszewski et al. 2008). PRL has been shown previously to act as a chemoattractant
for human breast carcinoma (Maus, Reilly et al. 1999) and to enhance the proliferation, motility and survival of breast cancer cells (LaPensee and Ben-Jonathan 2010). Recently, it was also found that women with higher circulating levels of PRL were more likely to fail treatment for breast cancer and had a lower survival rate (Tworoger and Hankinson 2008). In fact, exposing breast cancer cells to PRL prevents the cytotoxic effects of the chemotherapeutic drugs vinblastine, doxorubicin, taxol and cisplatin (LaPensee, Schwemberger et al. 2009). Many breast cancer cells are able to produce their own PRL, and it has been postulated that the locally-produced PRL acting in an autocrine/paracrine manner might be a factor contributing to the failure of previous attempts to treat breast cancer by inhibiting the synthesis and release of PRL from the pituitary (Clevenger 2003). These data suggest the importance of elucidating the mechanisms by which PRL mediates its effects on the development and persistence of breast cancer, in order to more effectively prevent and treat it.

**JAK2**

JAK2 is a member of the JAK family of tyrosine kinases, including JAK1, JAK2, JAK3 and TYK2. The JAK kinases contain a FERM domain at the N-terminus (also known as domains JH5-JH7) which is important for protein and receptor association along with regulation of catalytic activity (Jatiani, Baker et al. 2010), followed by an SH2 domain (comprised of domains JH3 and JH4) which does not bind phosphotyrosines (Higgins, Thompson et al. 1996; Kampa and Burnside 2000; Haan, Kreis et al. 2006), and a pseudokinase domain (JH2 domain) critical for negative regulation of the tyrosine kinase domain (JH1 domain) (Lindauer, Loerting et al. 2001; Saharinen, Vihinen et al. 2003).
JAK2 is a tyrosine kinase responsible for transmission of signals by two-thirds of the cytokine receptor superfamily, including receptors to interleukins, ciliary neurotrophic factor, growth hormone, prolactin, erythropoietin, granulocyte macrophage-colony stimulating factor, γ-interferon, leptin, leukemia inhibitory factor, and oncostatin M (O'Shea, Gadina et al. 2002). JAK2 molecules remain constitutively associated with the cytokine receptors, which change conformation upon ligand binding, promoting dimerization of the JAKs, allowing auto- and/or transphosphorylation of the JAK2 molecules and subsequent activation. Activation of JAK2 is followed by autophosphorylation of many tyrosines and the receptor associated with JAK2, providing docking sites for SH2- and PTB-containing domain proteins (Figure 2). Numerous proteins participate in signaling cascades downstream of JAK2 signaling, including signal transducers and activators of transcription (STATs), phosphatases, and adapter proteins Grb2, p85 phosphatidylinositol 3-kinase, She and Cbl, which lead to activation of the phosphatidylinositol 3-kinase/Akt and Ras/Raf/ERK pathways and stimulation of Fos and Jun transcriptional activity (Jatiani, Baker et al. 2010).

SH2B1β

SH2B1β is a 670 amino acid protein that was originally identified as a binding partner and substrate for the tyrosine kinase JAK2 (Rui, Mathews et al. 1997). SH2B1β is a member of the SH2B family of proteins, including SH2B1, SH2B2 (formerly known as APS) and SH2B3 (formerly known as Lnk). All members of the SH2B family contain N-terminal dimerization domains, several proline-rich regions, a pleckstrin homology domain and a SH2 domain. The SH2B1 gene encodes four splice variants (α, β, γ, δ), differing only in their C-termini (Nelms, O'Neill et al. 1999; Yousaf, Deng et al. 2001).
Figure 2. An overview of cytokine signaling. Cytokines bind to homodimeric or heterodimeric receptors, which are constitutively bound to Jaks. Jaks are thought to be activated by a conformational change in the receptor that allows trans- and/or auto-phosphorylation of the two bound Jaks. These in turn phosphorylate the cytokine receptors. Stat proteins bind the phosphorylated receptor chains, allowing the Jaks to phosphorylate the Stats. Phosphorylated Stats form dimers and translocate and accumulate in the nucleus, where they regulate gene expression. Reprinted from BioMed Central Ltd: [Genome Biology] K. Yamaoka, et al., Genome Biology (2004), 5:253. Copyright (2004).
SH2B1β has no known enzymatic function, but participates in both cytokine and growth factor signaling. Subsequent to cytokine stimulation, JAK2 binds to cytokine receptors, auto- and/or trans-phosphorylates, binds to SH2B1β and tyrosyl-phosphorylates SH2B1β. SH2B1β subsequently increases the tyrosine kinase activity of JAK2 and is phosphorylated by JAK2 on Tyr439 and Tyr494 (Rui and Carter-Su 1999; O'Brien, Argetsinger et al. 2003). The SH2 domain of SH2B1β binds to pTyr 813 of JAK2 (Kurzer, Argetsinger et al. 2004; Nishi, Werner et al. 2005), and is necessary for the maximal stimulation of JAK2 kinase activity (Rui, Gunter et al. 2000). Amino acids 269-555 of SH2B1β contain additional lower-affinity binding sites for JAK2 which are able to inhibit JAK2 when overexpressed without the SH2 domain of SH2B1β (Rui, Gunter et al. 2000). It is hypothesized that the association of JAK2 and SH2B1β through these lower-affinity binding domains is responsible for increasing local concentrations of SH2B1β near JAK2, assisting in binding of the SH2 domain to active JAK2. SH2B1β not only enhances the tyrosine kinase activity of JAK2, but also receptor tyrosine kinases including receptors to platelet derived growth factor (Rui and Carter-Su 1998; Riedel, Yousaf et al. 2000; Yousaf, Deng et al. 2001), insulin (Nelms, O'Neill et al. 1999; Riedel, Yousaf et al. 2000), insulin-like growth factor-I (Riedel, Yousaf et al. 2000; Yousaf, Deng et al. 2001), fibroblast growth factor (Kong, Wang et al. 2002), and nerve growth factor (Qian, Riccio et al. 1998; Rui, Herrington et al. 1999), suggesting that SH2B1β is important for a variety of cell functions. Knockout of the SH2B1 gene results in severe obesity and both leptin and insulin resistance, in addition to infertility, possibly due to resistance to insulin-like growth factor I (Ohtsuka, Takaki et al. 2002; Duan, Yang et al. 2004; Ren, Li et al. 2005). These studies support a role for SH2B1 as a positive regulator
of JAK2 signaling pathways initiated by leptin and insulin as well as potentially, insulin-like growth factor I.

In addition to serving as an adaptor protein near the plasma membrane with active receptor complexes, SH2B1β also acts as a nuclear protein. SH2B1β has been shown to undergo nucleocytoplasmic shuttling via its nuclear localization sequence (NLS) and nuclear export sequence (NES). Mutation of either the NLS or NES of SH2B1β is sufficient to inhibit SH2B1β-promoted NGF-dependent neuronal differentiation of PC12 cells, while mutation of only the NLS of SH2B1β is enough to impede NGF-dependent transcription of MMP-3 and -10, in addition to uPAR (Chen and Carter-Su 2004; Maures, Chen et al. 2009). These findings highlight the importance of SH2B1β function within the nucleus in promotion of neuronal differentiation. SH2B1β has also been shown to increase the cytoplasmic localization of transcription factor Foxo1, signifying a possible role for SH2B1β in regulation of gene expression through nucleocytoplasmic shuttling of transcription activators or repressors (Wang, Chen et al. 2004).

PAK1

PAK1 (p21-activated kinase) is a serine-threonine kinase activated by the small GTPases, Rac and Cdc42, and is implicated in the regulation of many biological functions, including cellular motility, survival, morphogenesis, division and malignant transformation (Bokoch 2003; Zhao and Manser 2005; Kumar, Gururaj et al. 2006). Activation of PAK1 kinase activity is regulated at multiple levels, involving the autoinhibition of the C-terminal catalytic domain by the N-terminal domain, along with dimerization and occupation of the catalytic cleft by association of the N-terminal and C-
terminal domains (Lei, Lu et al. 2000). PAK1 autoinhibition occurs in trans, when the inhibitory domain of a PAK1 molecule associates with the kinase domain of another PAK1 molecule (Parrini, Lei et al. 2002). Activation of the PAK1 molecule occurs upon binding of GTP-bound Cdc42 and Rac1 to the CRIB domain of PAK1, relieving the autoinhibitory conformation of PAK1, allowing autophosphorylation of PAK1 at Thr423, preventing the refolding and inhibition of PAK1 (Gatti, Huang et al. 1999; Chong, Tan et al. 2001). Signals transmitted via receptors to EGF, PDGF, VEGF and other receptor tyrosine kinases along with G protein-coupled receptors all activate PAK1 via Rac and Cdc42 (Dummler, Ohshiro et al. 2009) (Figure 3). PAK1 can also be activated by binding to Rac2, Rac3, TC10, Wrch-1 and CHP proteins (Manser, Leung et al. 1994; Aronheim, Broder et al. 1998; Knaus and Bokoch 1998; Neudauer, Joberty et al. 1998; Mira, Benard et al. 2000; Tao, Pennica et al. 2001). Although PAK1 localization is mainly cytoplasmic, PAK1 is also activated subsequent to its recruitment to the plasma membrane via Nck, PIX and Grb2 adapter proteins upon their activation by growth factor receptors (Bokoch, Wang et al. 1996; Lu, Katz et al. 1997; Daniels, Hall et al. 1998; Zhao, Manser et al. 2000). Phosphorylation of PAK1 at Thr423 (the same site autophosphorylated after activation of PAK1 by Cdc42 and Rac1) by PDK1 may be a key factor in recruitment of PAK1 to the plasma membrane by adapter proteins, or via activation of PAK1 by lipids such as sphingosine, which are able to activate PAK1 independent of GTPases (Bokoch, Reilly et al. 1998; King, Gardiner et al. 2000). Activation of PAK1 also occurs via phosphorylation of Ser21 by Akt1, which promotes disassociation of Nck with the N-terminus of PAK1, causing stimulation of PAK1 activity (Tang, Zhou et al. 2000; Zhao, Manser et al. 2000). Inhibition of PAK1 activity
Figure 3. Modes of PAK1 regulation in normal and cancer cells. A schematic representation showing multiple signalling pathways converging on the activation of PAK1 through both G-protein and tyrosine-kinase-dependent and -independent mechanisms. The well-characterized pathway for the activation of PAK1 is by the Rho GTPases, RAC1 and cell division cycle 42 (CDC42). Receptor tyrosine kinases (through growth factor receptor-bound protein 2 (GRB2) and NCK), non-receptor tyrosine kinases such as ETK, lipids, integrins and upstream serine/threonine kinases such as phosphatidylinositol 3 kinase (PI3K) and pyruvate dehydrogenase kinase isozyme 1 (PDK1) can also activate PAK1. Activation of PAK1 also occurs through the interaction of partners that release auto-inhibition by binding to PAK1. Activated PAK1 in turn initiates cascades of pathways that culminate in the cellular response. As a consequence of higher functional activity of Paks in tumours, either by virtue of increased expression or activity, cells acquire a distinct advantage and show characteristics that are associated with neoplastic transformation. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS CANCER] R. Kumar, et al., Nature Reviews Cancer (2006) 6, 459-471. Copyright 2006.
can occur through phosphorylation of PAK1 at Thr212, by the neuron-specific kinase Cdk5 (Nikolic, Chou et al. 1998; Rashid, Banerjee et al. 2001). In addition, PAK1 can be inactivated by binding of Pak-interacting protein (PIP) to the regulatory region of PAK1, eliminating PAK1 kinase activity (Xia, Ma et al. 2001). Merlin, a tumor-suppressor and product of the Neurofibromatosis gene, participates in negatively regulating PAK1 activation via binding directly to PAK1 and preventing Rac/Cdc42 binding and activation of PAK1 (Kissil, Wilker et al. 2003; Hirokawa, Tikoo et al. 2004; Xiao, Gallagher et al. 2005; Wilkes, Repellin et al. 2009). Phosphorylation of PAK1 at Thr212 by cyclin B-bound Cdc2 affects PAK1 protein-protein interaction but not activation (Banerjee, Worth et al. 2002; Thiel, Reeder et al. 2002).

PAK1 is involved in regulation of the actin and microtubule cytoskeletons. PAK1 localizes to various areas of the cortical actin cytoskeleton and the kinase activity of PAK1 contributes to directional cellular motility (Dharmawardhane, Sanders et al. 1997; Manser, Huang et al. 1997; Sells, Boyd et al. 1999; Sells, Pfaff et al. 2000). PAK1 also has numerous cytoskeletal-regulating substrates, including LIM kinase (Edwards, Sanders et al. 1999), p41-Arc (Vadlamudi, Li et al. 2004), vimentin (Chan, Kozma et al. 2002; Tang, Bai et al. 2005), MLCK (Sanders, Matsumura et al. 1999) and FLNa (Vadlamudi, Li et al. 2002), among others. Phosphorylation of LIMK on Thr508 by PAK1 contributes to the protrusiveness and persistence of the lamellipodia (Delorme, Machacek et al. 2007), while phosphorylation of p41-Arc is necessary for growth factor-dependent cell motility (Vadlamudi, Li et al. 2004). PAK1 phosphorylation of MLCK results in the inhibition of MLCK activity, leading to the subsequent deconstruction of
focal adhesions and stress fibers needed for cell motility (Sanders, Matsumura et al. 1999).

Aberrant PAK1 signaling and/or overexpression of PAK1 has been demonstrated in various cancers, including brain, liver, kidney, colon, bladder, ovarian, and breast (Dummler, Ohshiro et al. 2009). Notably, over 50% of human breast cancers exhibit PAK1 overexpression and/or hyperactivation (Balasenthil and Vadlamudi 2003). Several lines of evidence support a role for PAK1 in breast cancer. Activation of PAK1 by heregulin leads to an increase in invasion of breast cancer cells (Adam, Vadlamudi et al. 1998), while overexpression of kinase-dead PAK1 in extremely invasive breast cancer cells led to increased cell spreading, stabilization of stress fibers, and an overall reduction in invasiveness (Adam, Vadlamudi et al. 2000). In addition, hyper-activation of the PAK1 pathway in MCF7 cells promotes cell migration and anchorage-independent growth, though the cells are normally non-invasive (Vadlamudi, Adam et al. 2000). Some data suggest that mislocalization of PAK1 to focal adhesions in breast cancer cells leads to constitutive activation of PAK1 (Stofega, Sanders et al. 2004). PAK1 action on many of its cytoskeletal-associated substrates is one of the various ways PAK1 is thought to contribute to the motility and invasiveness of breast cancer cells. In addition, increased active PAK1 nuclear localization has been tied to the development of tamoxifen resistance in breast cancer (Holm, Rayala et al. 2006).

**Filamin A**

Filamin A (FLNa), a PAK1 substrate and binding partner, is a 280 kDa actin cross-linking protein and member of the Filamin family of proteins, including Filamin B
(FLNb) and Filamin C (FLNc). FLNa is the most widely expressed member, followed by FLNb (expressed in nonmuscle cells) and FLNc, which is expressed in adult cardiac and muscle tissues (Gorlin, Yamin et al. 1990; Takafuta, Wu et al. 1998; Xie, Xu et al. 1998; Xu, Xie et al. 1998; Thompson, Chan et al. 2000; Stossel, Condeelis et al. 2001; van der Flier and Sonnenberg 2001). All three Filamin family members contain an N-terminal actin-binding domain and a rod region containing 24 immunoglobulin-like repeats (Gorlin, Yamin et al. 1990; Stossel, Condeelis et al. 2001; van der Flier and Sonnenberg 2001; Pudas, Kiema et al. 2005). The last repeat of the rod region enables the filamin molecules to dimerize, allowing for a flexible structure mediating the actin gelation activity of filamins. It has been determined that filamins have >90 interacting partners, including adapter proteins, small GTPases, transmembrane receptors, and membrane channels (Nakamura, Stossel et al. 2011) (Figure 4). FLNa interacts with proteins in the nucleus, as well. Cleavage of FLNa between repeats 15 and 16 generates a fragment of FLNa (repeats 16-24) which has been shown to localize to the nucleus and downregulate androgen receptor functions, whereas full-length FLNa interacts with SEK-1, BRCA-2 and FOXC1 in the nucleus (Marti, Luo et al. 1997; Yuan and Shen 2001; Loy, Sim et al. 2003; Meng, Yuan et al. 2004; Berry, O’Neill et al. 2005). FLNa participates in the activation of various kinases as well as being regulated by kinases itself. FLNa is phosphorylated by several kinases at Ser2152, which is thought to act in modulating FLNa binding to integrins, along with rendering FLNa resistant to calpain cleavage (Vadlamudi, Li et al. 2002; Jay, Garcia et al. 2004; Woo, Ohta et al. 2004). FLNa binding to PAK1 enhances the kinase activity of PAK1, which subsequently phosphorylates FLNa at Ser2152, resulting in PAK1-dependent membrane ruffling (Vadlamudi, Li et al.
FLNa also stimulates PAK1 by interacting with sphingosine kinase 1, which phosphorylates sphingosine, leading to the direct activation of PAK1 (Maceyka, Alvarez et al. 2008).

As a potent actin cross-linking protein, FLNa is a major factor impacting the mechanics of cellular migration. One of the first noted defects of FLNa-deficient melanoma cells (M2 cells) was the inability to migrate due to inefficient polarization and continuous blebbing, which was rescued once FLNa was stably expressed (A7 cells) (Cunningham, Gorlin et al. 1992). FLNa mutations in humans lead to perturbed neuronal migration within the cerebral cortex, resulting in periventricular heterotopia (Fox, Lamperti et al. 1998). However, FLNa knockout mice do not develop periventricular heterotopia, nor do they have defective cellular migration, most likely because of compensation by FLN family members FLNb and FLNc (which is also expressed in nonmuscle cells during determined phases of development) (Feng, Chen et al. 2006; Hart, Morgan et al. 2006). FLNa also works with other proteins to mediate cytoskeletal rearrangements in order to regulate cell adhesion, spreading and migration. FLNa binds to Rho, Rac, Cdc42 and ROCK to modulate the cytoskeleton in various cell types (Ohta, Suzuki et al. 1999). FLNa interaction with CD28 is necessary for T-cell cytoskeletal remodeling, along with engagement of lipid microdomains and signaling molecules into the immunological synapse (Tavano, Contento et al. 2006). FLNa also assists in human immunodeficiency virus (HIV) infection by connecting HIV-1 receptors to the actin cytoskeleton regulating network (Jimenez-Baranda, Gomez-Mouton et al. 2007). Taken together, these data suggest that filamins function as scaffolds for numerous signaling pathways, bringing together many binding partners in order to unite receptors and
signaling molecules with the actin cytoskeleton, facilitating a wide variety of biological functions.
Figure 4. Monomeric structure of FLNA and its interaction partners. The actin-binding domain at the N terminus of filamin contains two calponin-homology domains depicted as CH1 and CH2. The 24 repeats that follow the actin-binding domain fold into antiparallel β-sheets and function as interfaces for protein–protein interactions. The majority of binding partners interact with FLNA at the C-terminus. The figure presents a selected collection of proteins that interact directly with FLNA. The approximate binding rod domain repeats involved in each interaction are also indicated. These partners are divided into groups of transcription factors, GTPase-related proteins, other filamins and filamentous actin, and cell adhesion and migration proteins. Reprinted by permission from Elsevier Inc.: [TRENDS IN CELL BIOLOGY] A-X. Zhou, et al., Trends in Cell Biology (2010) 20, 113-123. Copyright 2010.
II. JAK2 Phosphorylates PAK1 and PRL-dependent Phosphorylation of PAK1 Leads to Remodeling of the Actin Cytoskeleton

Abstract

The serine-threonine kinase, PAK1, participates in the regulation of many biological processes including cell survival, morphogenesis, angiogenesis, and regulation of the actin and microtubule cytoskeletons. PAK1 is regulated by GTPase-dependent and GTPase-independent mechanisms; however the function of tyrosyl phosphorylation in PAK1 regulation has not been fully elucidated. We have shown that JAK2 phosphorylates PAK1 in vivo and in vitro. In the current study we identify tyrosines 153, 201 and 285 in PAK1 as sites of JAK2 phosphorylation by two-dimensional peptide mapping and phosphoamino acid analysis. We demonstrate that prolactin activation of JAK2 and resulting phosphorylation of PAK1 significantly enhances cell membrane ruffling, cell motility and invasiveness of breast cancer cells. Taken together, our data suggest that JAK2 phosphorylates PAK1 at tyrosines 153, 201 and 285, and that this phosphorylation is necessary for maximal PRL-dependent motility and invasiveness of breast cancer cells.

Introduction

PAK1 is a serine-threonine kinase activated by a variety of GTPase-dependent and -independent mechanisms, leading to the PAK1-dependent modulation of a variety of biological processes, including regulation of the actin and microtubule cytoskeletons. PAK1 is required for heregulin-mediated membrane ruffling and participates in the regulation of cell motility and invasion (Adam, Vadlamudi et al. 1998). Regulation of
PAK1 has been extensively studied; however the role of tyrosyl phosphorylation of PAK1 has not been completely clarified, though 14 tyrosines exist within the PAK1 molecule. Previously it was determined that activation of PAK1 can occur via Etk/Bmx, a member of the Tec cytoplasmic tyrosine kinase family, which binds to and tyrosyl-phosphorylates PAK1 (Bagheri-Yarmand, Mandal et al. 2001). Furthermore, tyrosyl-phosphorylated active PAK1 was identified as a member of a Rho-dependent multiprotein complex in constitutively-active v-ErbB receptor-transformed cells (McManus, Boerner et al. 2000).

JAK2 is a tyrosine kinase responsible for transmission of signals from the majority of cytokine receptor family members, including the prolactin receptor (PRLR) (Maures, Kurzer et al. 2007). Upon PRL binding to its receptor, JAK2 becomes activated and phosphorylates the PRLR, in addition to autophosphorylating. The phosphorylated tyrosines in the PRLR and JAK2 act as docking sites for a variety of SH2- and PTB-domain containing proteins that localize to the active PRLR-JAK2 complexes, leading to a cascade of signaling pathways responsible for the various activities regulated by PRL. PRL is important for a wide variety of biological processes, in addition to being implicated in the development of breast cancer (Clevenger, Furth et al. 2003).

Clarifying how pathways downstream of JAK2 are mediated is critical to understanding the various mechanisms of JAK2 signaling. In our current study, we demonstrate that PRL-activated JAK2 phosphorylates PAK1. We have identified tyrosines 153, 201 and 285 in PAK1 as the sites of JAK2-dependent phosphorylation. We also demonstrate that PRL-dependent JAK2 phosphorylation of PAK1 is necessary
for maximal PRL-induced membrane ruffling, motility and invasion of breast cancer cells.

Materials and Methods

Plasmids - cDNAs encoding myc-PAK1 WT and HA-PAK1 WT were provided by Dr. Chernoff (Fox Chase Cancer Center, Philadelphia, PA); cDNAs encoding JAK2, JAK2 K882E, myc-SH2B1β, and myc-SH2B1β (504-670) were provided by Dr. Carter-Su (University of Michigan, Ann Arbor, MI; (Rui and Carter-Su 1999). Individual tyrosines in PAK1 were mutated to phenylalanines using the QuikChange site-direct mutagenesis kit (Stratagene). The double mutant, PAK1 Y(153,201)F was created by using PAK1 Y153F as a template and mutating Tyr 201. The triple mutant PAK1 Y(153, 201, 285)F (PAK1 Y3F) was created by using PAK1 Y(153,201)F as a template and mutating Tyr 285. Mutations were confirmed by sequencing by the University of Michigan DNA Sequencing Core. Construction of PAK1 WT in pLNCX2 retroviral vector containing IRES2-EGFP element was described previously (Li, Mullins et al. 2008). The final plasmids PAK1 WT and PAK1 Y3F with N-terminal myc-tags were expressed from retroviral constructs that include IRES elements that allow the transcription of a single bicistronic mRNA of myc-PAK1-IRES2-EGFP, and so produce myc-PAK1 together with EGFP as a reporter for expression of PAK1.

Antibodies – Monoclonal anti-phosphotyrosine (anti-PY, clone 4G 10) antibody (AB) and polyclonal anti-JAK2 (used for Fig. 2A) and rabbit anti-mouse from Upstate Biotechnology, Inc. (Millipore), monoclonal anti-HA from Covance, polyclonal anti-PAK N-20 and monoclonal anti-myc (9E10) from Santa Cruz Biotechnology, Inc.,
polyclonal anti-PAK1 from Cell Signaling were used for immunoprecipitation and immunoblotting. Monoclonal anti-JAK2 AB (#AHO1352, clone 691R5, Biosource) was used for immunoblotting.

Cells – 293T cells were purchased from American Type Culture Collection (ATCC) and grown in DMEM medium (Mediatech Cellgro) supplemented with 10 % calf serum (Hyclone), 1 mM glutamine, 100 units of penicillin per ml, 100 mg of streptomycin per ml. Phoenix cells, provided by Dr. William Taylor (University of Toledo, OH), were maintained at 37 °C in DMEM containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and supplemented with 4 mM l-glutamine, until 90% confluent. For transfection, the medium was changed to omit the antibiotics. Then, the cells were transfected with either pLNCX2-IRES2-EGFP, pLNCX2-myc-PAK1 WT-IRES2-EGFP, or pLNCX2-myc-PAK1 Y3F-IRES2-EGFP using a modification of the PEI method (Boussif, Lezouale'h et al. 1995). Briefly, the cDNA was added to plain DMEM, followed by addition of PEI at a ratio of 3:1 (µg PEI: µg cDNA), and the mixture was then incubated for 10 minutes at room temperature prior to addition to cells. Cells were incubated at 37 °C for 48 hours, after which the virus media was collected and centrifuged for 5 minutes at 200 g to remove cell debris and passed through a 0.45 µm filter. The supernatant media containing viruses was supplemented with polybrene at a final concentration of 12 µg/ml by the addition of a 4 mg/ml polybrene stock solution.

T47D breast cancer cells were provided by Dr. Ethier (Karmanos Cancer Institute, MI). T47D cells and their PAK1 clones were maintained in RPMI supplemented with 10% FBS (Sigma), bovine insulin (0.2 units/ml; Sigma-Aldrich) and antibiotics. TMX2-28 cells were a gift from Dr. Kathryn Eisenmann (University of Toledo, OH). TMX2-28
cells and their PAK1 clones were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and supplemented with 4 mM L-glutamine. To generate stable clones overexpressing GFP, PAK1 WT or PAK1 Y3F mutant, the viral media supplemented with polybrene was added to the cells at a ratio of 1:3 (viral broth to fresh medium) and cultured at 37 °C. The next day, the medium was removed and fresh complete medium was added to the cells. On the third day, the medium was changed again and replaced with fresh medium supplemented with 1.5 mg/ml G418 (Invivogen) to allow for selection of positive clones over a period of 2-3 months. Clonal cell lines were isolated and expanded and at least 6 clonal lines were examined for transgene expression by anti-myc immunoblot.

Phosphopeptide mapping and phosphoamino acid analysis - Wild type and mutant forms of HA-tagged PAK1 were co-expressed with JAK2 and SH21Bβ in 293T cells. The cells were deprived of serum overnight. PAK1 were immunoprecipitated with anti-HA and incubated with 0.5 mCi of [γ-32P]ATP at 30°C for 30 min in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography. The region of the nitrocellulose containing 32P-labeled PAK1 was excised, soaked in 500 μl of 0.5% polyvinylpyrrolidone in 100 mM acetic acid at 37 °C for 30 min and digested with 5 μg of methylated trypsin (Promega) for 4h at 37 °C. Digested peptides were lyophilized, oxidized in performic acid and re-lyophilized. Peptides were then separated first by thin layer electrophoresis and then in the second dimension by ascending chromatography (Boyle, van der Geer et al. 1991; O'Brien, Argetsinger et al. 2003). 32P-Labeling peptides were be visualized by autoradiography.
Assessment of membrane ruffling- To measure the effect of PAK1 mutants on membrane ruffling, T47D cells stably expressing GFP, WT PAK1 or PAK1 Y3F cells were deprived of serum and treated as indicated in the figure legends. Cells were rapidly rinsed three times with PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl) and fixed for 30 minutes at room temperature in 4% formaldehyde in PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min, rinsed three times with PBS and incubated with Texas Red phalloidin and located with a TRITC filter set using a Zeiss Axiovert 200 microscope. The number of ruffles per 100 cells per condition was determined. Each transfection was repeated at least three times with similar results.

Cell migration and invasion assays - In order to assess the effect of PAK1 mutants on migration, T47D cells stably expressing GFP, PAK1 WT or PAK1 Y3F were deprived, trypsinized, washed in PBS to removed trypsin and counted on a hemacytometer. Equal numbers of cells for each condition were placed in deprivation media in the upper chamber of a Boyden chamber (Corning). Each chamber was coated with collagen IV (Sigma; 1 µg/ml) on the underside of the filter. Deprivation media with or without 500 ng/ml hPRL was placed in the lower chamber. Cells were allowed to migrate for 48 hours, after which non-migrating cells were removed from the upper chamber by a cotton swab. Cells from five separate fields that had migrated through the pores of the membrane to the underside of the filter were counted after fixation and staining with propidium iodide and visualization by fluorescent microscopy.

To determine the effect of PAK1 tyrosyl phosphorylation on invasion, TMX2-28 cells stably expressing GFP, PAK1 WT or PAK1 Y3F were deprived, trypsinized, washed in PBS to remove trypsin and counted on a hemacytometer. Equal numbers of
cells for each condition were placed in deprivation media in the upper chamber of a Boyden chamber, which had been coated with Matrigel (BD Biosciences) on the underside of the filter. Deprivation media with or without 100 ng/ml hPRL was placed in the lower chamber. Cells were allowed to invade for 48 hours, after which non-invading cells were removed from the upper chamber by a cotton swab. Cells from five separate fields that had invaded the matrigel layer on the underside of the filter were counted after fixation and staining with propidium iodide and visualization by fluorescent microscopy.

Wounding assay - T47D cells stably expressing GFP, PAK1 or PAK1 Y3F were plated at high density onto tissue culture dishes coated with collagen IV (1 µg/ml). The next day, cells were deprived of serum for 24 hours. Following deprivation, monolayers of cells were scarified using a plastic pipette tip, washed extensively and incubated in deprivation media with or without 200 ng/ml hPRL. Ten measurements along each wound were taken using a phase-contrast microscope with a calibrated eyepiece at the initial time of wounding and 18 hours later, in order to calculate the percentage wound closure in 18 hours for each condition.

Results

We have seen that PAK1 is phosphorylated by JAK2, so in an effort to identify the PAK1 tyrosines phosphorylated by JAK2, we performed two-dimensional peptide mapping and phosphoaminoacid analysis. In order to perform two-dimensional peptide mapping, we first expressed HA-tagged WT PAK1 with WT JAK2 and myc-tagged WT SH2B1β in 293T cells, followed by overnight serum deprivation. JAK2 is constitutively active when it is overexpressed in 293T cells and SH2B1β strongly increases the tyrosine kinase activity of JAK2. We then used anti-HA to immunoprecipitate HA-PAK1-JAK2
complexes, and incubated the complexes with $[\gamma-^{32}\text{P}]\text{ATP}$ to allow phosphorylation of HA-PAK1 by JAK2. The protein complexes were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Bands containing $^{32}\text{P}$-labeled PAK1 were isolated following visualization by autoradiography and subjected to tryptic digestion. After tryptic digestion, we oxidized the peptides in performic acid and separated them in two dimensions by thin layer electrophoresis, followed by ascending chromatography. Autoradiography of the PAK1 phosphopeptide map exposed 24 phosphorylated peptides (Figure 5A, 6B). In an effort to determine which phosphopeptides contained phosphorylated tyrosines, we performed phoshoaminoacid analysis, for which all phosphopeptides were scraped and eluted, then hydrolyzed by acid. Of the 24 phosphorylated peptides, 3 contained phosphotyrosines (Fig. 5B, Spots 1, 2 and 3).

To ensure that these phosphorylated tyrosines were present as a consequence of JAK2 phosphorylation of PAK1, we performed two-dimensional peptide analysis using 293T cells expressing HA-tagged WT PAK1 without JAK2 and SH2B1β co-overexpression. The spots 1, 2, and 3 that had contained phosphotyrosines (Fig. 6B) were not present in this map (Fig. 6A). These observations suggest that the spots 1, 2 and 3 are sites of JAK2 tyrosine phosphorylation of PAK1.

In order to discover which tyrosines in PAK1 are phosphorylated by JAK2, we mutated each of the 14 PAK1 tyrosines to phenylalanine in HA-tagged WT PAK1 and performed two-dimensional peptide mapping. Phosphopeptide maps of each single tyrosine mutant of PAK1 were similar to that of WT PAK1, except in the cases of PAK1 Y153F, Y201F and Y285F mutants. Mutations in tyrosines 153, 201 and 285 resulted in the disappearance of spots 1, 2 and 3, respectively (Fig. 6C, D, E). Mutation of all 3
Figure. 5. Three tyrosyl-containing PAK1 phosphopeptides are phosphorylated by JAK2 in vitro. (A) Wild type PAK1 overexpressed in 293T cells with JAK2 was immunoprecipitated with anti-HA, incubated with 0.5 mCi of [γ-32P]ATP, resolved by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography. PAK1 band was cut from nitrocellulose and subjected to two-dimensional phosphopeptide mapping. (B) Phosphopeptides scraped from 2D map of PAK1 (A) were eluted and subjected to acid hydrolysis. Amino acids were separated by thin layer electrophoresis and visualized by autoradiography. Migration of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) standards is indicated. L. Rider and A. Shatrova et al., JBC (2007) 42, 30985-96.
Figure 6. JAK2 phosphorylates PAK1 at Tyr 153, 201 and 285. HA-PAK1 overexpressed without (A) or with JAK2 and SH2B1β (B-F) was IP’d with αHA and subjected to in vitro kinase assay and 2D phosphopeptide mapping. Spots 1, 2 and 3 were not present when HA-PAK1 overexpressed without JAK2 and SH2B1β. Spot 1 disappeared when Tyr 153 was mutated to phenylalanine (C). Spots 2 (D) and 3 (E) disappeared when Tyr(s) 201 and 285 were mutated to phenylalanines, respectively. Spots 1, 2 and 3 disappeared when all three Tyr(s) 153, 201 and 285 were mutated to phenylalanines (F). L. Rider and A. Shatrova et al., JBC (2007) 42, 30985-96.
tyrosines (PAK1 Y3F) revealed the disappearance of all 3 spots simultaneously (Fig. 6F). These results suggest that JAK2 phosphorylates PAK1 directly on tyrosines 153, 201 and 285 in vitro (Rider, Shatrova et al. 2007).

Previously it was demonstrated that PAK1 is required for maximal heregulin-dependent cell membrane ruffling (Adam, Vadlamudi et al. 1998). In order to examine how PRL-dependent JAK2 phosphorylation of PAK1 modulates actin cytoskeletal remodeling, we assessed ruffling in T47D breast cancer cells (which have endogenous prolactin receptor [PRLR] expressed) that we had stably expressed either GFP or GFP-containing PAK1 WT or PAK1 Y3F. T47D stable cells were deprived of serum and then treated with or without hPRL, fixed and stained with Texas Red Phalloidin. The ruffling index was determined by counting the total number of phalloidin-stained ruffles per 100 cells for each experimental condition by fluorescent microscopy. T47D cells stably-expressing WT PAK1 exhibited a significantly greater amount of PRL-stimulated membrane ruffling as compared to GFP-expressing T47D cells, while T47D cells stably-expressing PAK1 Y3F had a significantly lower level of PRL-mediated membrane ruffling when compared to T47D cells expressing PAK1 WT (Fig. 7). These results demonstrate that PRL-dependent JAK2 phosphorylation of PAK1 is necessary for the optimum PRL-induced membrane ruffling of T47D cells.

PAK1 has already been implicated in the regulation of cell motility and invasion (Kumar and Vadlamudi 2002; Vadlamudi and Kumar 2003; Kumar, Gururaj et al. 2006). To investigate whether JAK2 phosphorylation of PAK1 regulates migration of cells, we utilized the same T47D stable cells described above and assessed their ability to migrate into a wound. Monolayers of cells were scarified following serum deprivation and
Figure 7. Maximal PRL-dependent ruffling of T47D cells requires tyrosyl phosphorylation of PAK1. T47D cells stably expressing vector, PAK1 WT or PAK1 Y3F were serum deprived and treated with or without 200 ng/ml PRL for 15 min. Filamentous actin was visualized by staining with Texas Red Phalloidin (shown). Arrows indicate ruffles. Ruffling index as the number of ruffles per cell was counted and plotted in the graph. Bars represent mean +/- S.E. *, p<0.05, compared to cells expressing WT PAK and treated with PRL. Each experiment was performed three times and 100 cells were assessed for ruffling in each experiment.
treated with or without PRL. The percentage of wound closure was measured after 18 hours in the presence or absence of PRL. T47D cells stably expressing WT PAK1 had the highest percentage of wound closure in the presence of PRL, as compared to the control GFP T47D cells, while PAK1 Y3F-expressing T47D cells inhibited PRL-dependent migration of cells in the wound (Fig. 8). These results suggest that pTyr-PAK1 is required for the maximal PRL-induced wound closure in T47D cells.

To further elucidate how JAK2 phosphorylation of PAK1 regulates the PRL-induced migration of cells, we utilized the same T47D stable cells described above and performed a Boyden chamber migration assay. T47D stable cells were deprived, trypsinized, washed in PBS to remove trypsin and counted on a hemacytometer with a fluorescent microscope. Equal numbers of cells for each condition were placed in deprivation media in the upper chamber. Each chamber was coated with collagen IV on the underside of the filter. Deprivation media with or without 500 ng/ml PRL was placed in the lower chamber. PRL was used as a chemoattractant and activator of JAK2, stimulating JAK2 phosphorylation of WT PAK1. Cells were allowed to migrate for 48 hours, after which non-migrating cells were removed from the upper chamber by a cotton swab. Cells from five separate fields that had migrated through the pores of the membrane to the underside of the filter were counted after fixation, staining with propidium iodide and visualization by fluorescent microscopy (Fig. 9). Expression of WT PAK1 significantly enhanced the migration of T47D cells in response to PRL as compared to GFP expression alone, while expression of PAK1 Y3F significantly inhibited migration of cells in comparison to the GFP control cells (Fig. 9). These results
Figure 8. Maximal wound closure of T47D cells in response to PRL requires tyrosyl phosphorylation of PAK1. T47D cells stably expressing GFP, WT PAK1 or PAK1 Y3F mutant were deprived of serum. Following deprivation, monolayers of cells were scarified and treated with (black bars) or without (white bars) 200 ng/ml PRL. The percentage wound closure in 18h was calculated for each condition. Bars represent mean +/- S.E. *, p<0.05 compared to cells expressing GFP and treated with PRL.
Figure 9. Maximal migration of T47D cells in response to PRL requires tyrosyl phosphorylation of PAK1. T47D cells stably expressing GFP, PAK1 WT or PAK1 Y3F were serum deprived, and equal amounts of cells were loaded into the upper part of the Boyden chamber. The number of cells that migrated to the lower surface of the chamber, toward deprivation media (white bars) or toward PRL (500 ng/ml) (black bars), after 48 h were counted in five random fields and plotted. Bars represent mean +/- S.E. *, p<0.05 compared to cells expressing GFP and treated with PRL.
suggest that phosphorylation of PAK1 by JAK2 is required for the maximal PRL-dependent migration of T47D cells.

To examine how JAK2 tyrosine phosphorylation of PAK1 modulates cell invasiveness, we utilized the TMX2-28 cell line instead of the non-invasive/minimally invasive T47D cell line. The TMX2-28 cell line is a highly motile and invasive clonal derivative of the MCF7 breast cancer cell line, which has PRLR endogenously expressed, and is therefore a useful model in studying the role of pTyr-PAK1 in PRL-stimulated cell invasion. We made TMX2-28 cells stably expressing GFP, or GFP-containing WT PAK1 or PAK1 Y3F and assessed their ability to invade Matrigel (a basement membrane complex). TMX2-28 stable cells were counted following deprivation and trypsinization and loaded in equal numbers onto Boyden chambers that were coated on the underside with Matrigel. Deprivation media with or without 100 ng/ml human PRL was placed in the lower chamber. Cells were allowed to invade for 48 hours, after which non-invading cells were removed from the upper side of the Boyden chamber by a cotton swab. Cells from five separate fields that had invaded the matrigel layer on the underside of the filter were counted after fixation and staining with propidium iodide and visualization by fluorescent microscopy. TMX2-28 cells expressing WT PAK1 and GFP were significantly more invasive than cells expressing GFP alone in response to PRL, while expression of PAK1 Y3F inhibited PRL-stimulated invasion (Fig. 10). Taken together, these observations suggest that tyrosine phosphorylation of PAK1 by JAK2 is required for maximal invasion of TMX2-28 cells in response to PRL.
Figure 10. Maximal invasion of breast cancer cells TMX2-28 (clone of MCF-7 cells) in response to PRL requires tyrosyl phosphorylation of PAK1. TMX2-28 cells stably expressing GFP, PAK1 WT, or PAK1 Y3F were serum deprived, and equal amounts of cells were loaded into the upper part of the Boyden chamber and PRL (100 ng/ml) was added to the lower chamber which was covered with Matrigel. The number of GFP-positive cells that migrated to the lower surface of the chamber in the presence (black bars) or absence (white bars) of PRL and invaded the Matrigel after 48 h were counted in five random fields. Bars represent mean +/- S.E. *, p<0.05 compared to cells expressing GFP and treated with PRL.
Discussion

We have demonstrated that PAK1 is Tyr-phosphorylated by JAK2 and that Tyr 153, Tyr 201 and Tyr 285 are the major sites of JAK2 phosphorylation. We were able to conclude this as a result of our two-dimensional phosphopeptide mapping experiments. Phosphopeptide maps of JAK2-tyrosyl-phosphorylated PAK1 from in vitro kinase assays demonstrated that JAK2 phosphorylation of PAK1 occurred on three of 21 phosphopeptides, as most of the phosphopeptides were present in the absence of JAK2. Of the 21 phosphorylated peptides present in the PAK1 map made in the presence of JAK2, only three contained phosphoTyr in addition to phosphoSer and phosphoThr when phosphoamino acid analysis was performed. All of the phosphopeptides contained either Ser, Thr or both (data not shown), whether or not JAK2 was present. PAK1 is a serine-threonine kinase which is autophosphorylated and phosphorylated by other serine-threonine kinases (Kumar, Gururaj et al. 2006). As we expected, when either Tyr 153, 201 or 285 of PAK1 were mutated to phenylalanine, one of the spots on the phosphopeptide map disappeared, and mutation of Tyr 153 and 201 simultaneously led to the disappearance of two spots, while mutation of all three Tyr 153, 201 and 285 led to the elimination of all three spots at once. Mutating any of the other remaining Tyr in PAK1 did not change the PAK1 phosphopeptide map. An increase of $^{32}$P incorporation into some of the Ser and Thr spots and the appearance of new Ser/Thr spots when PAK1 and JAK2 were co-expressed is likely due to the increase in PAK1 kinase activity from JAK2 tyrosyl phosphorylation of PAK1.

There has been previous evidence supporting a role for pTyr-PAK1 in various contexts. For example, the cytoplasmic tyrosine kinase Etk/Bmx of the Tec family of
non-receptor tyrosine kinases associates with and phosphorylates PAK1, subsequently activating PAK1 (Bagheri-Yarmand, Mandal et al. 2001). Additionally, in constitutively activated v-Erb receptor-transformed cells, a very active and tyrosyl-phosphorylated form of PAK1 was found (McManus, Boerner et al. 2000). Another PAK family member, PAK2, is regulated by tyrosyl phosphorylation. Src kinases phosphorylated PAK2 at Tyr 130 (Renkema, Pulkkinen et al. 2002). In contrast, another non-receptor tyrosine kinase, c-Abl, phosphorylates PAK2, but causes a decrease in PAK2 kinase activity. Therefore, our findings taken together with previously published data suggest that tyrosyl phosphorylation of PAK1 plays an important role in regulating PAK1 actions and elicits different responses in PAK1 dependent on specific extracellular signals. Taking this into consideration, our data demonstrate that PAK1 is a novel member of the JAK2 signaling pathway and that JAK2 directly phosphorylates PAK1 on tyrosines 153, 201 and 285.

Another major function of PAK1 is in modulating cytoskeletal reorganization and cell motility. We have demonstrated that JAK2 tyrosyl phosphorylation of PAK1 is required for the maximal ruffling of T47D cells in response to PRL, as stable expression of PAK1 WT led to a significant augmentation in PRL-induced membrane ruffling, while PAK1 Y3F stable expression failed to enhance ruffling. We have also shown, through wound healing and Boyden chamber assays, that the PRL-dependent motility of T47D cells is maximal in the presence of pTyr-PAK1. When JAK2 could no longer phosphorylate PAK1, as when PAK1 Y3F was expressed in T47D cells, both the PRL-dependent wound closure and migration of cells were inhibited, suggesting that PRL-induced activation of JAK2 and subsequent tyrosyl phosphorylation of PAK1 is required for maximal cell motility. In further support of a role for pTyr PAK1 in PRL-dependent
regulation of the cytoskeleton, we have also demonstrated that JAK2 phosphorylation of PAK1 is necessary for the maximal invasion of TMX2-28 cells. Inhibition of PRL-mediated invasion occurred in the presence of PAK1 Y3F, but was greatest when PAK1 WT was expressed. Taken together, these data provide strong evidence for JAK2 tyrosyl phosphorylation of PAK1 having a major role in regulating PRL-dependent cytoskeletal rearrangement. PAK1 has many actin-regulating substrates, including LIM kinase (Edwards, Sanders et al. 1999), p41-Arc (Vadlamudi, Li et al. 2004) and filamin (Vadlamudi, Li et al. 2002). It is possible that JAK2 tyrosyl phosphorylation of PAK1 causes an enhancement of PAK1 kinase activity, leading to an increase in phosphorylation of one or more of these substrates, resulting in cytoskeletal modulation. Another possible explanation of the motility-promoting effects of pTyr-PAK1 is that JAK2 tyrosyl phosphorylation of PAK1 affects the ability of PAK1 to find, bind and/or phosphorylate its substrates, thereby enhancing the effects of PAK1 on motility and invasion. The phosphorylated tyrosines in PAK1 could also serve as docking sites for SH2 domain-containing proteins, recruiting additional signaling molecules to the active JAK2-pTyr PAK1 complexes. Further study is needed in order to characterize targets that may bind to PAK1 at pTyr 153, 201 and/or 285.

In summary, using two-dimensional phosphopeptide mapping, we have identified Tyr(s) 153, 201 and 285 in PAK1 as sites of JAK2 phosphorylation. We demonstrated that JAK2 Tyr–phosphorylation is required for the maximal PRL-induced motility- and invasion-promoting effects of PAK1. Thus, our results introduce PAK1 as a novel JAK2 target in PRL-dependent signaling and elucidate the possible mechanism by which JAK2 and PAK1 participate in cell motility and invasion.
III. Adapter Protein SH2B1β Cross-links Actin Filaments and Regulates the Actin Cytoskeleton

Abstract

The widely-expressed SH2 domain-containing adapter protein SH2B1β was initially identified as a binding partner and substrate of JAK2 tyrosine kinase. SH2B1β is a member of the SH2B family (SH2B (SH2B1), APS (SH2B2), and Lnk (SH2B3)), which contains a conserved N-terminal dimerization, central pleckstrin homology, and C-terminal SH2 domains. Deletion of the SH2B1 gene results in severe obesity and both leptin and insulin resistance, as well as infertility, suggesting that SH2B1β may play a fundamental role in cell functions. SH2B1β has also been previously implicated in the regulation of the actin cytoskeleton by growth hormone and PDGF, and cell motility. SH2B1β is also required for maximal actin-based motility of Listeria monocytogenes. SH2B1β increases the rate of bacteria propulsion in infected cells and in cell extracts in a VASP-dependent fashion. However, the mechanisms by which the effects of SH2B1β on the actin cytoskeleton are mediated remain unknown. Here we extend initial findings identifying SH2B1β as a participant in the regulation of the actin cytoskeleton by showing that SH2B1β has two actin-binding sites: amino acids 150-200 that strongly bind to F-actin and amino acids 615-670 that bind F-actin less strongly, as assessed by high speed cosedimentation assay. Using a low speed pelleting assay and electron microscopy, we showed that SH2B1β cross-links actin filaments in vitro. In cells, SH2B1β localizes not only to cell ruffles as previously shown but also along the filopodia. Deletion of amino acids 150-200 leads to mislocalization of the mutant protein to the filopodia "tip complexes" where the mutant co-localizes with VASP. Using VASP-
deficient MVD7-/- cells and the same cells stably re-expressing GFP-VASP, we showed that proper intracellular localization of SH2B1β depends on the presence of the first actin-binding site and the presence of VASP. Finally, we demonstrate that both actin-binding domains of SH2B1β are required for maximal growth hormone-induced cell ruffling and for cell motility as assessed by phagokinetic assay. We hypothesize that SH2B1β functions as an adapter protein that in response to JAK2 activation, cross-links actin filaments leading to modulation of cellular responses.

**Introduction**

SH2B1β is an adaptor protein that participates in cytokine signaling, and is a binding partner and substrate for the tyrosine kinase JAK2 (Rui, Mathews et al. 1997). SH2B1β increases the tyrosine kinase activity of JAK2 and is tyrosyl phosphorylated by JAK2 (Rui and Carter-Su 1999). SH2B1β also participates in regulation of the actin cytoskeleton. SH2B1β enhances growth hormone- and platelet derived growth factor-stimulated ruffling and pinocytosis (Herrington, Diakonova et al. 2000). SH2B1β associates with the small GTPase, Rac, through the N-terminal proline-rich region of SH2B1β, promoting cytoskeletal rearrangement, and is required for maximal cell motility (Diakonova, Gunter et al. 2002). SH2B1β is also necessary for the maximal actin-based motility of the pathogen, Listeria monocytogenes. SH2B1β works in cooperation with vasodilator-stimulated phosphoprotein (VASP) to accelerate the movement of L. monocytogenes in cell extracts and infected cells (Diakonova, Helfer et al. 2007).

Additional members of the SH2B family have also been shown to regulate the actin cytoskeleton. F-actin assembly was reduced in mast cells from SH2B2 knockout mice, along with increased degranulation, signifying the importance of SH2B2 in regulating
actin during the process of degranulation (Kubo-Akashi, Iseki et al. 2004). SH2B2 was colocalized with F-actin during B-cell receptor capping in SH2B2 transgenic B cells (Iseki, Kubo et al. 2004). SH2B2 binds to Enigma, a protein involved in insulin-mediated actin rearrangement, and Vav3, a guanine nucleotide exchange factor for Rac (Yabana and Shibuya 2002; Barres, Gonzalez et al. 2005; Barres, Gremeaux et al. 2006). Another SH2B family member, SH2B3, binds directly to the actin cross-linking protein, FLNa (He, Li et al. 2000).

Here we extend initial findings identifying SH2B1β as a participant in the regulation of the actin cytoskeleton by showing that SH2B1β has two actin-binding sites: amino acids 150-200 that strongly bind to F-actin and amino acids 615-670 that bind F-actin less strongly. We demonstrate that cellular localization of SH2B1β is dependent on the first actin-binding site and the presence of VASP, and that SH2B1β utilizes both actin-binding domains in order to cross-link actin filaments. Finally, we show that both actin-binding domains are required for maximal growth hormone- and PRL-induced cell ruffling as well as for maximal cell motility.

**Materials and Methods**

Plasmids, antibodies and cells - cDNAs encoding myc-tagged and GST-tagged SH2B1β, were described previously (Rui and Carter-Su 1999; Rui, Gunter et al. 2000). To generate a series of truncated SH2B1β mutants, restriction sites were inserted at appropriate positions in myc-tagged or GST-tagged SH2B1β by using a QuickChange site-directed mutagenesis kit (Stratagene). Mutations were verified by DNA sequencing. cDNA encoding GFP-VASP was described previously (Geese, Loureiro et al. 2002). Porcine growth hormone and human prolactin were purchased from the National
Hormone and Peptide Program, NIDDK, Dr. Parlov. Polyclonal antibodies (AB) raised against SH2B1 (Rui, Mathews et al. 1997) were provided by Dr. Carter-Su and used for immunoprecipitation and immunoblotting. αJAK2 serum was provided by Dr. Carter-Su (Argetsinger, Campbell et al. 1993) and used for immunoprecipitation, monoclonal αJAK2 AB (#AHO1352, clone 691R5, Biosource) and αphosphotyrosine (αPY, clone 4G10; Upstate biotechnology, Inc.) were used for immunoblotting. Anti-myc (Santa Cruz Biotechnology, Inc.), phalloidin-rhodamine, phalloidin-AlexaFluor488, phalloidin-AlexaFluor 647 (Invitrogen) were used for immunocytochemistry. The stocks of mouse 3T3 F442A fibroblasts were provided by Dr. Green (Harvard University, Cambridge, MA). The MVD7 -/- cells derived from embryonic fibroblasts taken from MENA/VASP double knockout mice and MVD7 -/- re-expressing GFP-VASP (Bear, Cell, 2000) were provided by Dr. Gertler (Massachusetts Institute of Technology, Boston, MA). T47D cells were provided by Dr. Ethier (Karmanos Cancer Institute, Detroit, MI).

Immunocytochemistry - For localization studies, 3T3 F442A, MVD7 -/- or MVD7 -/- cells stably expressing GFP-VASP, were transfected with cDNA encoding myc-tagged versions of SH2B1β and GFP-VASP if indicated using the Amaza method (Amaza Inc.) according to the manufacturer’s protocol. The cells were replated on coverslips and processed for immunocytochemistry. The coverslips were fixed (Diakonova, Gunter et al. 2002) and incubated with αmyc followed by goat-αmouse-AlexaFluor 594 or goat-αmouse-AlexaFluor 488 (Invitrogen). Staining by secondary antibody reagent alone was negligible (not shown). The actin was stained by either phalloidin-rhodamine, phalloidin- AlexaFluor 488 or phalloidin-AlexaFluor
Confocal imaging was performed with an Olympus 1X70 laser scanning confocal microscope.

Actin preparation and cosedimentation assay - Rabbit skeletal muscle actin (Cytoskeleton Inc.) was subjected to an actin polymerization-depolymerization cycle prior to use in experiments. G-actin was polymerized by addition of 50 mM KCl, 2 mM MgCl₂ and 1 mM ATP. After polymerization, F-actin was centrifuged at 150,000g for 90 min, the pellet was resuspended in G-buffer (2 mM Tris-HCl pH 8.5, 0.2 mM CaCl₂, 0.2 mM ATP, and 0.5 mM DTT), and dialyzed overnight against G-buffer at 4 °C. Next, G-actin was spin-clarified at 150,000g for 1 hour to remove degraded actin. G-actin was polymerized as above. Following polymerization, the F-actin was centrifuged at 150,000g for 90 min, and the pellet was resuspended in F-buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl₂, 1 mM ATP and 0.2 mM CaCl₂), including 0.05% sodium azide, and stored at a concentration of 140 μM at 4 °C. For experiments with EGTA, F-actin after polymerization was resuspended and dialyzed against an EGTA-G-buffer (2 mM Tris-HCl pH 8.0, 0.1 mM ATP, 0.2 mM EGTA, 0.05 mM MgCl₂ and 0.1 mM DTT), polymerized as described above, pelleted and finally resuspended in EGTA-F-buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl₂, 1 mM ATP and 0.2 mM EGTA).

GST or GST-tagged WT SH2B1β or SH2B1β mutants were purified using a glutathione-agarose affinity column (Sigma-Aldrich). The purity of the eluted proteins was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to performing cosedimentation assays, GST-tagged WT SH2B1β or SH2B1β mutants were centrifuged at 150,000g for 2 hours to remove any insoluble
protein aggregates. For cosedimentation assays, increasing amounts (0.5 μM to 28 μM) of GST-tagged WT SH2B1β or mutants were incubated with 4 μM F-actin for 30 min at 24 °C in F-buffer, and then sedimented at 150,000g for 2 hours at 24 °C. Supernatants were separated from pellets, and pellets were resuspended in F-buffer. Equivalent amounts of supernatant and pellet fractions were subjected to SDS-PAGE. Gels were stained with Coomassie blue, and protein bands were quantified using Bio-Rad Quantity One software. The amount of WT GST-SH2B1β or GST- SH2B1β mutants bound to F-actin was calculated from the known concentrations of proteins in the assay and the ratios of each protein in the supernatant and pellet. The results were plotted to indicate the concentration of WT GST-SH2B1β or GST- SH2B1β mutants versus amount bound to 4 μM F-actin.

Low speed centrifugation - For low speed pelleting assay, 0.1 μM GST-SH2B1β WT or GST- SH2B1β mutants were incubated with 8 μM F-actin for 30 minutes at 24 °C in F-buffer, and then sedimented at 16,000g for 1 hour at 24 °C. Supernatants were separated from pellets, and pellets were resuspended in F-buffer. Both supernatant and pellet fractions were subjected to SDS-PAGE. Gels were stained with Coomassie blue, and actin bands were quantified as described above. The results were plotted to indicate the percentage of actin bundled (pellet fraction) or not bundled (supernatant fraction) for each GST, GST-SH2B1β or GST- SH2B1β mutant.

Electron microscopic analysis - To test actin bundling /cross-linking activity, 15 μM F-actin was incubated with either 1 μM GST-SH2B1β, 1 μM GST-SH2B1β (Δ-Δ), or 1 μM GST in F-buffer for 30 min at 24 °C. Following centrifugation at 16,000g for 1 hour at 24 °C, pellets, if present, were dissolved in F- buffer and immediately applied to
poly-lysine coated glow-discharge carbon grids. If no pellet was present, the centrifuged mixture was applied to the aforementioned grids. The actins on grids were fixed with 2.5% glutaraldehyde and stained with 1% phosphotungstic acid, each for 1 min. Electron microscopy was performed with a Zeiss EM10 electron microscope at 60 KV.

Assessment of membrane ruffling - To measure the effect of SH2B1β mutants on membrane ruffling, cells expressing the indicated proteins were deprived of serum and treated as indicated in the figure legends. Cells were rapidly rinsed three times with PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl) and fixed for 30 min at room temperature in 4% formaldehyde in PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min, rinsed three times with PBS and incubated with αmyc followed by goat αmouse- AlexaFluor 488. F-actin was stained with phalloidin-rhodamine. Transfected cells expressing myc-tagged forms of SH2B1β were located with a FITC filter set using a Zeiss Axiovert 200 microscope. The number of ruffles per transfected cell was determined. Only cells with similar levels of GFP or myc-tagged indicated forms of SH2B1β expression were scored. Each transfection was repeated at least three times with similar results.

Phagokinetic assay - For the phagokinetic assay 3T3 F442A cells were transfected with cDNA encoding indicated proteins using the Amaza method (Amaza, Inc.) and plated on colloid gold-covered coverslips 24 hours after transfection (Albrecht-Buehler 1977; Diakonova, Gunter et al. 2002). Cells remove particles while they move, thereby producing areas that are free of colloid gold. The incubation time (7 hours) was experimentally determined to avoid overlapping of the particle-free areas produced by neighbor cells. After 7 hours the coverslips were fixed with 4% paraformaldehyde for 30
min, permeabilized with 0.1% Triton X-100 for 15 min, and incubated with αmyc followed by goat αmouse- AlexaFluor 488. F-actin was stained with phalloidin-rhodamine. Individual transfected cells were located with a FITC filter set using a Zeiss Axiovert 200 microscope. Only cells with similar levels of GFP or myc-tagged indicated forms of SH2B1β expression were scored. Differential interference contrast (DIC) images were collected and particle-free areas were quantified using Image Tool software. The particle-free area was measured in three independent experiments. The phagokinetic index was calculated as a ratio of particle-free to cross-sectional area of the cell. The images presented in the figure are representative of three independent experiments.

Results

SH2B1β binds directly to F-actin (Diakonova, Helfer et al. 2007). In an effort to characterize the actin-binding region(s) of SH2B1β, we overexpressed various myc-tagged truncation mutants of SH2B1β along with GFP-tagged actin and performed co-immunoprecipitation experiments (Fig. 11). From these data, we determined that amino acids 150-200 and 615-670 of SH2B1β contained putative actin-binding sites. In order to identify whether amino acids 150-200 and 615-670 of SH2B1β bind directly to actin, various truncation mutants of SH2B1β were expressed as GST fusion proteins and tested for their ability to bind F-actin in a high speed co-sedimentation assay. Increasing concentrations of GST, GST- WT SH2B1β or GST- SH2B1β mutants were incubated with a fixed concentration of F-actin (4 µM) and subjected to centrifugation of 150,000 x g for 2 hours. At this speed, soluble proteins will pellet only if they bind directly to F-actin. To ensure that sedimentation of proteins occurred due to direct binding to F-actin and not self-aggregation, control centrifugations with GST, GST- WT SH2B1β or GST-
Figure 11. SH2B1β binding to actin requires amino acids 150-200 and 615-670. 293T cells overexpressing the indicated proteins were immunoprecipitated with αmyc and immunoblotted with αGFP. Cell lysates were immunoblotted with αGFP for actin and αmyc for forms of SH2B1β. The migration of GFP-actin and the various myc-tagged forms of SH2B1β are indicated on the right. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
SH2B1β mutants alone were performed (Fig.12). GST- WT SH2B1β was detected in the pellet fractions, confirming direct binding to F-actin as determined previously (Fig.12B). GST alone was unable to pellet with F-actin, suggesting that the GST tag remaining on each SH2B1β construct did not affect binding to F-actin. Deletion of a putative N-terminal actin-binding domain, SH2B1β (Δ150-200) mutant, led to a decrease in the ability of SH2B1β to bind F-actin, as compared to WT SH2B1β (Fig.12B and C). A mutant containing only AA 150-200, SH2B1β (150-200), was able to bind better to F-actin than WT SH2B1β (Fig. 12F). Co-sedimentation of F-actin with SH2B1β (Δ150-200, Δ615-670), a mutant lacking both putative actin-binding domains, revealed that SH2B1β was no longer able to bind F-actin when both sites were removed (Fig.12E), suggesting that AA150-200 and AA615-670 are the domains of SH2B1β responsible for direct binding of SH2B1β to F-actin. This result was confirmed with the co-sedimentation of F-actin with SH2B1β (615-670), demonstrating that AA615-670 are another actin-binding domain and that this domain binds less strongly to F-actin than AA150-200 (Fig.12).

Actin-binding proteins that contain more than one actin-binding site often arrange actin filaments into organized structures such as bundles, cross-linked networks or gels. In order to examine whether SH2B1β might utilize both actin-binding domains to manipulate actin filaments, we incubated F-actin with GST, GST- WT SH2B1β or GST-SH2B1β mutants and performed low-speed centrifugation assays. At low speed (16,000 x g), actin will only pellet if it has been aggregated by the protein incubated with it. Centrifugation of actin with GST- WT SH2B1β resulted in pelleting of actin at low speed, whereas GST and GST-tagged mutants of SH2B1β were unable to pellet actin at
Figure 12. SH2B1β binds F-actin.
Prior to each experiment, WT SH2B1β and mutants were centrifuged in the absence of F-actin to remove any insoluble protein aggregates. A defined concentration of F-actin (4 μM) was mixed with increasing concentrations of the forms of SH2B1β or GST alone. Ratios above gel images indicate concentrations of recombinant proteins (in μM) to the constant concentration of F-actin (4 μM). After ultracentrifugation at 150,000×g for 2h, equivalent amounts of pellet (P) and supernatant (S) fraction were subjected to SDS-PAGE followed by Coomassie blue staining (A-G, left column). Protein bands were quantified using Bio-Rad Quantity One software. The amount of WT GST-SH2B1β or GST-SH2B1β mutants bound to F-actin was calculated from the known concentrations of proteins in the assay and the ratios of each protein in the supernatant and pellet. Plots of concentration (X axis) vs. amount bound (Y axis) for WT SH2B1β and all mutants are shown in the right column. Plots of concentration (X axis) vs. amount bound (Y axis) for WT SH2B1β and deletion mutants (H) and two actin-binding site mutants (J) are shown together. Bars represent mean +/- S.E., n=3. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
low speed (Fig.13A, B). Incubation of actin with both actin-binding domains of SH2B1β added together resulted in actin remaining in the supernatant fraction (Fig. 13A, B), suggesting that both actin-binding domains of SH2B1β present in an intact SH2B1β molecule are required in order for SH2B1β to aggregate actin in vitro.

In order to determine the nature of the SH2B1β-mediated actin aggregates, electron microscopy was performed. Actin aggregates were examined based upon an established set of criteria from Harris et al. (Harris, Rouiller et al. 2006), in which bundles are characterized by filaments arranged along their long axes and a cross-linked network as an array of non-parallel filaments. In the presence of GST alone, actin was present in only single filaments (Fig.13C). Addition of GST- SH2B1β WT caused the formation of a cross-linked actin network, which was significantly reduced when both actin-binding domains were deleted, in the case of GST- SH2B1β (Δ150-200, Δ615-670) (Fig.13C). These data indicate that SH2B1β is an F-actin cross-linking protein.

In vivo, SH2B1β localizes to cell membrane ruffles (Herrington, Diakonova et al. 2000). In order to study the role of both actin-binding domains on the localization of SH2B1β, myc-tagged WT SH2B1β or SH2B1β actin-binding domain mutants were expressed in 3T3 F442A cells and immunofluorescence microscopy was performed. WT SH2B1β and SH2B1β Δ150-200, Δ615-670, Δ-Δ, 150-200 and 615-670 mutants all localized in membrane ruffles and along the length of filopodia (Fig.14 A-F). However, the Δ150-200 and Δ-Δ mutants also localized to the tips of filopodia, a phenomenon not observed with WT SH2B1β or any of the other SH2B1β actin-binding domain mutants (Fig.14B, D). These observations indicated that the first actin-binding site of SH2B1β (AA150-200) is necessary for the proper localization of SH2B1β. The filopodium tip
Figure 13. SH2B1β cross-links actin filaments. For the low speed centrifugation assay (A, B), 0.1 μM GST-SH2B1β WT or GST- SH2B1β mutants were incubated in with 8 μM F-actin for 30 min in actin polymerizing buffer (F-buffer), and then sedimented at 16,000g for 1h at 24°C. (A) Equivalent amounts of pellet (P) and supernatant (S) fraction were subjected to SDS-PAGE followed by Coomassie blue staining. (B) Actin bands were quantified and plotted to indicate the percentage of actin bundled/cross-linked (pellet fraction, black bar) or not bundled/cross-linked (supernatant fraction, grey bar) for each GST-SH2B1β or GST- SH2B1β mutant. Bars represent mean +/- S.E. (C) Electron micrographic images of negatively stained F-actin in the presence of GST (left), SH2B1β (middle) and Δ-Δ mutant (right). L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
Figure 14. Intracellular localization of SH2B1β depends on both the first actin-binding domain of SH2B1β and VASP protein. (A-F) 3T3 F442A cells overexpressing the indicated forms of myc-SH2B1β were stained with α-myc (green) and phalloidin-rhodamine (red). Long arrows indicate filopodia and short arrows indicate ruffles. The boxed regions were enlarged and merged. Asterisks denote the tips of filopodia. Scale bars, 20 μm. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
Figure 14 (continued). Intracellular localization of SH2B1β depends on both the first actin-binding domain of SH2B1β and VASP protein. (G-I) 3T3 F442A cells overexpressing GFP- VASP (green) and the indicated forms of myc-SH2B1β, were stained with α-myc (red), and phalloidin-647 (blue). Long arrows indicate filopodia and short arrows indicate ruffles. The boxed regions were enlarged and merged. Asterisks denote the tips of filopodia. Scale bars, 20 μm. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
Figure 14 (continued). Intracellular localization of SH2B1β depends on both the first actin-binding domain of SH2B1β and VASP protein. (J-L) MVD7 -/- cells overexpressing the indicated forms of myc-SH2B1β were stained with α-myc (red), and phallloidin-488 (green). (M-O) MVD7 -/- stably overexpressing GFP-VASP (green) were transfected with cDNA encoding the indicated forms of myc-SH2B1β, stained with α-myc (red), and phallloidin-647 (blue). Long arrows indicate filopodia and short arrows indicate ruffles. The boxed regions were enlarged and merged. Asterisks denote the tips of filopodia. Scale bars, 20 μm. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
complex is composed of many proteins, some of which have yet to be characterized, but includes members of the Ena/VASP family of proteins (Lanier, Gates et al. 1999; Rottner, Behrendt et al. 1999). In order to determine whether SH2B1β (Δ150-200) and SH2B1β (Δ-Δ) were indeed localizing to the filopodium tip complex, GFP-VASP was co-expressed in 3T3 F442A cells with WT SH2B1β, Δ150-200, or Δ-Δ, to act as an indicator of the tip complex. Upon analysis via immunofluorescence microscopy, it was determined that SH2B1β Δ150-200 and Δ-Δ co-localized with GFP-VASP at the tips of filopodia, in contrast to WT SH2B1β, which did not (Fig.14G-I). These data confirm that AA150-200 of SH2B1β are responsible for the proper localization of SH2B1β, and that deletion of these amino acids results in mislocalization of SH2B1β to filopodium tip complexes.

Previous data demonstrated that SH2B1β localization in the actin tails of Listeria is VASP-dependent (Diakonova, Helfer et al. 2007). In order to examine the role of VASP on SH2B1β intracellular localization, immunofluorescence microscopy was performed in VASP-deficient MVD7 -/- cells expressing either myc-tagged WT SH2B1β or SH2B1β actin-binding site mutants. WT SH2B1β exhibited impaired localization in ruffles and patchy localization in filopodia when expressed in MVD7 -/- cells, as compared to 3T3 F442A cells (Fig.14J). Both Δ150-200 and Δ-Δ localized along the length of filopodia in MVD7 -/- cells, in contrast to WT SH2B1β, but did not localize to the tips of filopodia, as we had previously seen in 3T3 F442A cells (Fig.14K,L).

Expression of WT SH2B1β, Δ150-200 and Δ-Δ in MVD7 -/- cells with GFP-VASP stably re-expressed revealed restored localization of WT SH2B1β in cell membrane ruffles and along the length of filopodia, while the Δ150-200 and Δ-Δ mutants once again
localized in filopodia tips, along with GFP-VASP (Fig. 14M-O). Taken together, these data indicate that the proper intracellular localization of SH2B1β depends upon the presence of VASP and the first actin-binding site of SH2B1β (AA150-200).

SH2B1β was previously implicated in growth hormone (GH) - dependent ruffling (Herrington, Diakonova et al. 2000). In order to determine the physiological role of SH2B1β-actin binding on cytoskeletal rearrangement, we overexpressed myc-tagged WT SH2B1β and SH2B1β actin-binding site mutants in 3T3 F442A cells and analyzed membrane ruffling in response to GH treatment. Overexpression of WT SH2B1β resulted in significantly enhanced GH-dependent membrane ruffling as compared to cells expressing GFP as control (Fig.15), in agreement with previous work (Herrington, Diakonova et al. 2000). In contrast, cells expressing SH2B1β Δ150-200, Δ615-670, 150-200 or 615-670 mutants exhibited ruffling comparable to the GFP-expressing cells, whereas expression of SH2B1β Δ-Δ was able to inhibit GH-stimulated cell ruffling (Fig.15). These observations suggest that SH2B1β Δ-Δ acts as a dominant-negative protein, and that both actin-binding domains of SH2B1β are required for maximal GH-dependent membrane ruffling in 3T3 F442A cells.

In an effort to further characterize the biological relevance of SH2B1β-actin binding on cytokine-dependent cytoskeletal remodeling, we examined PRL-stimulated membrane ruffling in T47D cells. Cells overexpressing myc-tagged WT SH2B1β exhibited the greatest amount of PRL-dependent ruffling as compared to cells expressing GFP as control, whereas overexpression of myc-tagged SH2B1β Δ150-200, Δ615-670, 150-200 or 615-670 mutants neglected to increase PRL-stimulated cell ruffling (Fig.16). In addition, myc-tagged SH2B1β Δ-Δ operated as a dominant-negative and inhibited
Figure 15. Both actin-binding sites of SH2B1β are required for maximal growth hormone-induced membrane ruffling. (A - F) 3T3 F44A cells expressing GFP or the indicated forms of myc-tagged forms of SH2B1β were serum-deprived (A, C, E) and treated with 500 ng/ml GH for 10 min (B, D, F). Filamentous actin was visualized by staining with phalloidin-rhodamine (shown) and the transfected cells were visualized by staining with α-myc. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 50 μm. (G) Ruffling index as the number of ruffles per cell was counted. Bars represent mean +/- S.E. *, p<0.05 compared to cells expressing GFP and treated with GH. Δ150-200 and Δ615-670 are designated as Δ150 and Δ615, respectively. Each experiment was repeated three times, 100 cells were assessed for ruffling in each experiment for each type of transfection. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
Figure 16. Actin-binding sites of SH2B1β are involved in PRL-induced membrane ruffling. (A - F) T47D cells expressing GFP or the indicated forms of myc-tagged forms of SH2B1β were serum-deprived (A, C, E) and treated with 150 ng/ml PRL for 15 min (B, D, F). Filamentous actin was visualized by staining with phalloidin-rhodamine (shown) and the transfected cells were visualized by staining with α-myc. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 50 μm. (G) Ruffling index as a number of ruffles per cell was counted. Bar represents mean +/− S.E. *, p<0.05 compared to cells expressing GFP and treated with PRL. Δ150-200 and Δ615-670 are designated as Δ150 and Δ615, respectively. Each experiment was repeated four times, 100 cells were assessed for ruffling in each experiment for each type of transfection. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
Figure 17. Maximal phagokinesis of 3T3 F442A cells requires SH2B1β. (A-F) 3T3 F442A cells expressing GFP or the indicated forms of myc-tagged SH2B1β were plated on colloid gold-covered coverslips and the areas that became free of colloid gold in 7 hrs were visualized (outlined). The cells overexpressing GFP or the indicated forms of myc-SH2B1β were stained with α-myc and phalloidin-rhodamine (not shown). Individual transfected cells were located with a FITC filter set (B, D, F) and then observed by DIC (A, C, E). Scale bar, 20 μm. (G) A phagokinetic index was calculated as the ratio of the particle-free area to the cross-sectional area of the cells. Bars represent mean +/- S.E. * p<0.05 compared to cells expressing GFP. Δ150-200 and Δ615-670 are designated as Δ150 and Δ615, respectively. n= 111, 247, 153, 159, 95, 85, and 63 for cells expressing GFP, WT, Δ150-200, Δ 615-670, Δ-Δ, 150-200 and 615-670 of SH2B1β, respectively. L. Rider, et al., Mol. Endocrinology (2009) 23, 1065-76.
PRL-dependent ruffling (Fig.16), suggesting that both actin-binding sites of SH2B1β are necessary for maximum enhancement of PRL-stimulated membrane ruffling in T47D cells.

In order to determine whether SH2B1β-actin binding might affect cellular motility, we assessed the phagokinetic index of 3T3 F442A cells overexpressing myc-tagged WT SH2B1β, Δ150-200, Δ615-670, Δ-Δ, 150-200, 615-670, or GFP as control. The phagokinetic index of 3T3 F442A cells was calculated by measuring the area of colloid gold particles that were removed by transfected cells after seven hours, in ratio to the cross-sectional area of the cell, and plotted (Fig.17). Expression of Δ150-200, Δ615-670, Δ-Δ, 150-200 or 615-670 resulted in an inhibition of cellular motility, as compared to cells expressing GFP or WT SH2B1β. This data suggests that SH2B1β plays a role in cellular motility and that the actin-binding site mutants of SH2B1β can impede this aspect of SH2B1β function.

Discussion

Prolactin and growth hormone are just a couple of the various cytokines that initiate actin cytoskeletal remodeling (Wiedermann, Reinisch et al. 1993; Goh, Pircher et al. 1997; Maus, Reilly et al. 1999; Herrington, Diakonova et al. 2000; Reddy, Pushpanathan et al. 2007). Actin is responsible for carrying out a multitude of different functions in the cell, including cell division, migration, membrane ruffling and even signal transduction. Actin and its associated proteins, therefore, play an important role in mediating cytokine and growth factor signaling. Adapter proteins serve as an important bridge between the cytoskeleton and signal transduction components. Adapter proteins
have no enzymatic function, but work by bringing proteins together. The SH2B family of proteins participates in multiple signaling pathways (Maures, Kurzer et al. 2007). One member of the SH2B family, SH2B1β, has already been implicated in connecting the actin cytoskeleton to JAK2 signaling, as SH2B1β is necessary for GH-dependent cell ruffling and pinocytosis (Herrington, Diakonova et al. 2000), as well as maximum cellular motility (Diakonova, Gunter et al. 2002). SH2B1β also participates in the regulation of *Listeria monocytogenes* motility (Diakonova, Helfer et al. 2007). SH2B1β has already been shown to bind actin (Diakonova, Helfer et al. 2007) and associate with Rac1 (Diakonova, Gunter et al. 2002). Here we report for the first time that SH2B1β has two actin binding domains: amino acids 150-200 on the N-terminus that bind actin with a much greater ability than the second, C-terminal actin binding domain, amino acids 615-670. We also show that SH2B1β cross-links actin filaments in vitro, via low speed pelleting assay and electron microscopy. Previous findings support these data, as SH2B1β localizes with actin in tails of *Listeria* (Diakonova, Helfer et al. 2007), cell ruffles (Herrington, Diakonova et al. 2000) (and here), filopodia (here) and lamellipodia *in vivo*. *Listeria* actin tails have a similar dendritic-type array of actin fibers, made by the Arp 2/3 complex, like that found in lamellipodia, not stress fibers. Therefore, that we observed exclusion of SH2B1β from stress fibers, but localization in filopodia and lamellipodia, agrees with the finding of SH2B1β localization along *Listeria* tails and in filopodia and lamellipodia. Filopodia are actin-enriched formations made up of many actin-binding proteins and filamentous actin that participate in signal transduction, embryonic development, wound healing, adhesion, metastasis and chemoattractant guidance (Gupton and Gertler 2007; Mattila and Lappalainen 2008). Filopodia formation
is a controversial subject, as two different mechanisms involving different sets of actin-regulating proteins have been suggested (Svitkina, Bulanova et al. 2003; Pellegrin and Mellor 2005). In light of this, our finding that the actin-crosslinking protein SH2B1β localizes in filopodia could be of great interest. Additionally, we show that SH2B1β filopodia localization is dependent on VASP, because in VASP-deficient MVD7-/- cells SH2B1β localization was drastically different. Restoration of VASP in the same cells through stable re-expression resulted in normal SH2B1β localization. However, when the N-terminal actin-binding domain of SH2B1β was removed (Δ150-200 and Δ-Δ mutants), SH2B1β mutants mislocalized to the “tip complexes” of filopodia with VASP. Although not all members of the tip complex have been identified, those that have been include Ena/VASP proteins (Lanier, Gates et al. 1999; Rottner, Behrendt et al. 1999), myosin X (Berg and Cheney 2002), Dia2 (Schirenbeck, Bretschneider et al. 2005; Yang, Czech et al. 2007) and some others (Small, Stradal et al. 2002; Gupton and Gertler 2007; Mattila and Lappalainen 2008). Why is it that SH2B1β co-localizes with VASP in tip complexes when the N-terminal actin-binding domain is removed? The SH2B1β N-terminus has several proline-rich regions, including a FPSPP sequence (amino acids 12-16), which is similar to the FPPPP VASP-binding motif (Niebuhr, Ebel et al. 1997). The Listerial protein ActA has several copies of the FPPPP motif that bind Ena/VASP family members (Smith, Theriot et al. 1996; Niebuhr, Ebel et al. 1997; Machner, Urbanke et al. 2001). It has been shown that prolines in the core of the motif can be swapped for other amino acids (Niebuhr, Ebel et al. 1997), however the first proline is required for VASP binding (Ball, Kuhne et al. 2000). SH2B1β does not directly bind to VASP, but may associate indirectly through other proteins, like ActA in Listeria (Diakonova, Helfer et al.
2007). Our hypothesis is that the N-terminal actin-binding domain (amino acids 150-200) may somehow act to mask the FPSPP proline-rich motif in SH2B1β, impeding VASP binding. VASP binding is allowed once this binding domain is removed from SH2B1β (mutants Δ150-200 and Δ-Δ), which leads to VASP-mediated relocalization of SH2B1β to tip complexes. If VASP does indeed bind directly to the FPSPP sequence of SH2B1β when the first actin-binding domain of SH2B1β is deleted remains to be established. Another possibility is that the N-terminal actin-binding site of SH2B1β also modulates the correct intracellular localization of SH2B1β.

Prior studies have suggested that SH2B1β participates in membrane ruffling and motility (Herrington, Diakonova et al. 2000; Diakonova, Gunter et al. 2002). In this study, we have expanded upon the previous findings and identified two actin-binding sites in SH2B1β that are necessary for maximum GH- and PRL-dependent ruffling and motility. The phenomena of cell ruffling occurs in a multitude of different cell types as a result of stimulation by different extracellular stimuli and on moving cells, where ruffles are thought to be needed for directional cell motility. Therefore, cell ruffling may be a result of increased cell motility in response to a stimulus (Ridley 1994; Borm, Requardt et al. 2005). Our observation that SH2B1β acts as a dominant negative when one or both actin-binding domains is deleted suggests that wild type SH2B1β participates in actin cross-linking in GH- and PRL-dependent membrane ruffling and motility of cells. We hypothesize that GH or PRL binding to receptor relocates SH2B1β to activated receptor-JAK2 complexes where it is able to cross-link actin filaments and mediate cell ruffling and migration. Tyrosyl phosphorylation of SH2B1β by JAK2 could also create additional binding sites for
SH2 domain-containing proteins that may enhance cell ruffling in response to ligand binding. In support of this, it has been shown that JAK2-phosphorylated tyrosines 439 and 494 of SH2B1β are involved in GH-dependent ruffling (O'Brien, Argetsinger et al. 2003). We are in the process of identifying actin-binding proteins that associate with tyrosyl-phosphorylated SH2B1β. PRL has already been identified as a chemoattractant for breast carcinoma, but how PRL mediates its effects on cytoskeletal remodeling is not known (Maus, Reilly et al. 1999). Our findings might shed light on one of the ways in which PRL might assist in metastasis of breast cancer. PRL activation leads to association of active JAK2 with SH2B1β, which then binds to and cross-links actin filaments, via its two actin-binding domains, leading to maximum cell ruffling and migration. Growth hormone also enhances migration of various types of cells (Wiedermann, Reinisch et al. 1993) and our current research may enlighten how this process unfolds, as well. In addition, data from studies performed in Dictyostelium, Drosophila and vertebrates suggest that the JAKs pathways are necessary for an unusually wide array of developmental choices including cell migration (Hou, Zheng et al. 2002). Whether SH2B1β and its actin cross-linking activity participate in this process remains to be evaluated.
IV. Adapter Protein SH2B1β Binds Filamin A to Regulate Prolactin-Dependent Cytoskeletal Reorganization and Cell Motility

Abstract

SH2B1β is an adaptor protein that participates in both cytokine and growth factor signaling and is involved in regulation of the actin cytoskeleton. SH2B1β is a binding partner and substrate for the tyrosine kinase JAK2, increases the tyrosine kinase activity of JAK2, and promotes cytoskeletal rearrangement, inducing membrane ruffling and cellular motility. We have shown previously that SH2B1β contains two F-actin binding domains, and that these domains are required to enhance growth hormone (GH) and prolactin (PRL)-dependent membrane ruffling, as well as increase cell motility. Here we report that Filamin A (FLNa) is a novel binding partner of SH2B1β. We demonstrate that endogenous SH2B1β associates with endogenous FLNa in vivo, and that SH2B1β binds directly to FLNa through repeats 17-23 of FLNa and AA200-260 of SH2B1β in vitro. Utilizing FLNa-deficient M2 cells and the same cells stably re-expressing FLNa (A7 cells), we have determined that FLNa and the presence of both actin-binding domains of SH2B1β are required for maximal PRL-stimulated cell ruffling. Previously we have shown that another FLNa binding partner - serine-threonine kinase PAK1 – also participates in PRL-induced ruffling. JAK2 tyrosine kinase is activated by PRL and directly phosphorylates three tyrosines on the PAK1 molecule, and these three tyrosines are required for maximum PRL-dependent ruffling. In conclusion, we show that the actin- and FLNa-binding deficient (SH2B1β 3Δ) mutant of SH2B1β eliminated both PRL-mediated ruffling and PRL-dependent cell migration when co-expressed with PAK1 Y3F (JAK2 tyrosyl-phosphorylation deficient mutant). We verify the importance of the
relationship of SH2B1β, PAK1 and FLNa in PRL-mediated migration of breast cancer cells. Taken together, these findings demonstrate how PRL activation of JAK2 leads to regulation of the actin cytoskeleton and cellular motility through Filamin A, PAK1 and SH2B1β. We propose a model for PRL-dependent regulation of the actin cytoskeleton that integrates our findings with previous studies.

Introduction

Prolactin (PRL), secreted by the pituitary, participates in over 300 different biological processes, including stimulation of lactation, for which it is named (Bernichtein, Touraine et al. 2010). PRL is also produced by various organs such as the mammary gland, prostate, skin, brain, immune cells and adipocytes, among others. Recent data suggest that PRL may contribute to mammary tumorigenesis by enhancement of metastasis, increase in cell proliferation and survival (Clevenger, Furth et al. 2003). PRL not only acts as a chemoattractant in breast carcinoma but was recently shown to enhance the motility of breast cancer cells through Nek3 and Vav2, via activation of Rac and phosphorylation of paxilin (Miller, DeMaria et al. 2005; Miller, Antico et al. 2007). Recent studies in animals also support the involvement of PRL in cancer metastasis and tumor development (Liby, Neltner et al. 2003). Previously we have shown that SH2B1β increases PRL-dependent JAK2 tyrosyl phosphorylation (Rider, Tao et al. 2009). SH2B1β was originally identified as a binding partner and substrate of JAK2. SH2B1β participates in multiple cytokine-initiated signaling cascades and has previously been implicated in regulation of the actin cytoskeleton (Rui, Mathews et al. 1997). Other members of the SH2B1 family of proteins have been identified as participants in actin reorganization dynamics, in addition to associating with actin-
regulating proteins. SH2B3 (formerly known as Lnk) binds the actin cross-linking protein FLNa (He, Li et al. 2000). Recently, we identified two F-actin binding domains in the SH2B1β molecule and demonstrated that these actin-binding domains are necessary for SH2B1β to cross-link actin filaments, and that this is required for maximal PRL-induced membrane ruffling (Rider, Tao et al. 2009).

PAK1, a serine-threonine kinase, is another PRL-dependent substrate of JAK2 (Rider, Shatrova et al. 2007). PAK1, which is activated by Cdc42 and Rac1 in addition to other proteins, modulates actin and microtubule cytoskeletons and has been implicated in breast cancer (Dummler, Ohshiro et al. 2009). PAK1 has many substrates that are actin-regulating proteins, including myosin light chain kinase (Sanders, Matsumura et al. 1999), paxillin (Turner, Brown et al. 1999), Filamin A (Vadlamudi, Li et al. 2002), merlin (Kissil, Wilker et al. 2003) and p41-Arc (Vadlamudi, Li et al. 2004). Recently, we showed that PAK1 is a novel substrate of JAK2 tyrosine kinase, and that upon PRL administration, JAK2 phosphorylates PAK1 in vivo. The PAK1 tyrosines that JAK2 phosphorylates are 153, 201 and 285, which we identified by mass spectrometry and two-dimensional peptide mapping. Our data suggest that JAK2 phosphorylation of PAK1 at these three tyrosines is critical for cell survival and cell motility (Rider, Shatrova et al. 2007).

PAK1 directly phosphorylates Filamin a (FLNa) at serine 2152 and FLNa then increases both the autophosphorylation and kinase activity of PAK1 (Vadlamudi, Li et al. 2002). FLNa is an actin-binding and cross-linking protein which connects actin filaments to the plasma membrane (Stossel, Condeelis et al. 2001). Since the SH2B family member SH2B3 was identified as a binding partner for FLNa (He, Li et al. 2000) and
PAK1 directly phosphorylates FLNa (Vadlamudi, Li et al. 2002), we hypothesized that FLNa might associate with both SH2B1β and JAK2 tyrosyl-phosphorylated PAK1, and subsequently participate in the motility of breast cancer cells downstream of PRL.

In our current study, we show that SH2B1β is a FLNa-binding protein. We identify amino acids 200-260 of SH2B1β as the FLNa-binding domain, and repeats 17-23 of FLNa as the SH2B1β-binding domain. The SH2B1β-FLNa association is important for PRL-mediated actin remodeling. We also demonstrate that JAK2 phosphorylation of the three tyrosines of PAK1 as well as the presence of FLNa participate in PRL-dependent membrane ruffling. In conclusion, we show that the actin- and FLNa-binding deficient (SH2B1β 3Δ) mutant of SH2B1β eliminated both PRL-mediated ruffling and PRL-dependent cellular membrane ruffling along with PRL-dependent cell migration when co-expressed with PAK1 Y3F (JAK2 tyrosyl-phosphorylation deficient mutant). We verify the importance of the relationship of SH2B1β, PAK1 and FLNa in PRL-mediated migration of breast cancer cells.

Materials and Methods

Plasmids - cDNAs encoding myc-tagged WT SH2B1β, SH2B1β (1-105) or SH2B1β Δ-Δ mutant were described previously (Rui and Carter-Su 1999; Diakonova, Gunter et al. 2002; Rider, Tao et al. 2009). To generate myc-tagged SH2B1β (1-200) and (1-260) mutants, restriction sites were inserted at appropriate positions in myc-tagged WT SH2B1β by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). mRFP-tagged SH2B1β WT and SH2B1β Δ-Δ mutant were generated using the mRFP plasmid as a gift of Dr. Pugacheva (West Virginia University, Morgantown, WV).
To generate mRFP-tagged SH2B1β 3Δ, restriction sites were inserted at appropriate positions in the mRFP-tagged SH2B1β Δ-Δ mutant by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by sequencing at the University of Michigan DNA Sequencing Core. Schematic representations of WT and mutant forms of rat SH2B1β used in the study is shown in Figure 18. cDNAs encoding myc-tagged PAK1 WT and PAK1 Y3F mutant in which tyrosines 153, 201 and 285 in PAK1 WT were mutared to phenylalanines were described previously (Rider, Shatrova et al. 2007). cDNA encoding the GFP-containing long form of PRL receptor was described previously (Kline, Roehrs et al. 1999; Zheng, Koblinski et al. 2008). cDNAs encoding GST-tagged truncated forms of Filamin A were also described previously (Cukier, Li et al. 2007).

Cells - FLNa-deficient human melanoma cell line (M2) and its derivative line (A7), which stably expresses FLNa were described previously (Cunningham, Gorlin et al. 1992). M2 cells were maintained in MEM (Mediatech) containing 10% FBS (Sigma-Aldrich), 1% non-essential amino acids (Hyclone), 50 U/ml penicillin (Mediatech), 50 μg/ml streptomycin (Mediatech) and supplemented with 4 mM L-glutamine (Mediatech). A7 cells were maintained in the same medium as M2 cells but additionally supplemented with 500 μg/ml G418 (Invivogen). HEK 293T cells were maintained in DMEM (Mediatech) containing 10% calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and supplemented with 4 mM L-glutamine. T47D cells were maintained in RPMI (Mediatech) supplemented with 10% FBS, bovine insulin (0.2 units/ml; Sigma-Aldrich), 50 U/ml penicillin, 50 μg/ml streptomycin and supplemented with 4 mM L-glutamine. To generate stable T47D cell clones overexpressing PAK1 WT and PAK1 Y3F mutant, the
Figure 18. Schematic representation of wild type and mutant forms of rat SH2B1β used in the study. Actin-binding domains (amino acids 150-200 and 615-670) are shown in grey. FLNa-binding domain (amino acids 200-260) is shown in black. PH is the plekstrin homology domain (amino acids 274-376) and SH2 is the SH2 domain (amino acids 527-620). Proline-rich regions (amino acids 13-24, 89-103, 469-496), and dimerization domain (amino acids 24-85) are not shown. L. Rider and M. Diakonova, *Mol. Endocrinology*, in press.
T47D cells were transfected with a construction of PAK1 WT in pLNCX2 retroviral vector containing IRES2-EGFP element (Li, Mullins et al. 2008). To make PAK1 Y3F construct, tyrosines 153, 201 and 285 in PAK1 WT were mutated to phenylalanines using the QuikChange site-directed mutagenesis kit (Stratagene) (Rider, Shatrova et al. 2007).

Immunocytochemistry - For localization studies, M2 and A7 cells were transfected with cDNA encoding GFP-PRLR, and mRFP-tagged either SH2B1β WT or 3Δ using Nucleofector kit V (Lonza) according to the manufacturer’s instruction. The cells were replated on coverslips and processed for immunocytochemistry (Diakonova, Gunter et al. 2002). The coverslips were fixed and incubated with phalloidin-AlexaFluor 647 for actin visualization. Confocal imaging was performed with an Olympus 1X70 laser scanning confocal microscope.

Assessment of membrane ruffling - Transient transfections were performed using Nucleofector kit V (Lonza) according to the manufacturer’s instruction. The cells were replated on coverslips and processed for immunocytochemistry (Diakonova, Gunter et al. 2002). To measure the effect of SH2B1β and PAK1 mutants on membrane ruffling, M2 and A7 cells expressing the indicated proteins were deprived of serum (deprivation media is MEM medium supplemented with 1% BSA (Millipore), 50 U/ml penicillin, 50 μg/ml streptomycin and 4 mM L-glutamine) and treated as indicated in the figure legends. The coverslips were incubated with αmyc (Santa Cruz) followed by goat αmouse-AlexaFluor594 (Invitrogen) in the case of myc-PAK1 only transfection. F-actin was stained with AlexaFluor350-phalloidin (Invitrogen). When both myc-PAK1 and mRFP-SH2B1β were transfected, myc-PAK1 was stained with αmyc, followed by rabbit-αmouse and goat αrabbit- AlexaFluor350 (Invitrogen). The number of ruffles per
transfected cell was determined using a Zeiss Axiovert 200 microscope. Confocal imaging was performed with an Olympus 1X70 laser scanning confocal microscope. Each transfection was repeated at least three times with similar results.

Wounding Assay - M2 and A7 cells were transfected with cDNA encoding the indicated proteins using the Nucleofector kit V (Lonza) according to the manufacturer’s instruction. After 24 hours, cells were replated at high density (190,000 M2 cells/well and 95,000 A7 cells/well) onto 24-well plates. After 1 day, cells were deprived of serum for 24 hours. Following deprivation, monolayers of cells were scarified using a plastic pipette tip, washed extensively and incubated in deprivation media with or without 400 ng/ml hPRL (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases (Dr. Parlow)). Ten measurements along each wound were taken using a phase-contrast microscope with a calibrated eyepiece at the initial time of wounding and 8 hours later, in order to calculate the percentage wound closure in 8 hours for each condition. For some experiments, T47D cells stably expressing either GFP or GFP-containing PAK1 or PAK1 Y3F were transiently transfected with cDNA encoding indicated proteins using the Nucleofector kit V (Lonza) and processed as above.

In vitro binding assay - GST or GST-tagged FLNa truncated mutants were purified using a glutathione-agarose affinity column (Sigma-Aldrich). The purity of the proteins was monitored by SDS-PAGE. Myc- or mRFP-tagged WT or truncated forms of SH2B1β were translated in vitro using TNT Coupled Reticulocyte Lysate System T7 or SP6 (Promega) with or without 35S-methionine according to the manufacturer’s instruction. The mixture of GST-tagged FLNa mutants and in vitro translated SH2B1β mutants was rotated for 1 h or overnight at 4°C. The glutathione-agarose beads were
washed, and bound myc-SH2B1β was detected by IB with αmyc and bound mRFP-SH2B1β mutant was detected by autoradiogram. Amount of GST or GST-FLNa mutants was detected by IB with αGST.

Co-immunoprecipitation and immunoblotting - SH2B1β was immunoprecipitated from 293T cell lysates using αSH2B1β (Rui, Mathews et al. 1997) and protein A-agarose. Proteins were resolved by SDS-PAGE followed by immunoblotting with αFLNa (Cell Signaling Inc.). The same blot was reprobed with αSH2B1β. M2 and A7 cells were transiently transfected with cDNA encoding indicated proteins using the Nucleofector kit V (Lonza) and processed as described in the figure legends.

Results

Upon growth factor or hormone stimulation, cells initially respond by producing actin-rich membrane ruffles. These ruffles are generated at the leading edge of cells, and assumed to be necessary for motility (Ridley 1994). Both FLNa and SH2B1β have been demonstrated to mediate cell ruffling. SH2B1β participates in growth hormone-, PDGF- and PRL-induced membrane ruffling and both its actin-binding domains are necessary for optimum ruffling (Herrington, Diakonova et al. 2000; Rider, Tao et al. 2009). FLNa is crucial for heregulin –induced ruffling (Vadlamudi, Li et al. 2002). In order to determine how both FLNa and SH2B1β participate in PRL ruffling, we utilized the M2 cell line, a human melanoma cell line deficient in FLNa, and the A7 cell line, which is the same line but with FLNa stably re-expressed (Cunningham, Gorlin et al. 1992). Both lines have endogenous JAK2 and SH2B1β, though not PRLR, so we overexpressed a GFP-containing human long PRLR in both A7 and M2 cell lines and demonstrated that
deprivation eradicates tyrosyl-phosphorylated JAK2 while PRL administration activates JAK2 (Fig. 19).

To assess the role of FLNa and SH2B1β in PRL-dependent cytoskeletal remodeling, we examined PRL-dependent membrane ruffling in M2 and A7 cell lines. Myc-tagged SH2B1β WT or SH2B1β Δ-Δ mutant (both actin-binding domains removed) and GFP-PRLR were co-expressed in M2 and A7 cells and treated with PRL. The ruffling index was determined by counting the total number of phalloidin-containing ruffles per cell. Both M2 and A7 cells generate membrane ruffles upon treatment with PRL when expressing GFP-PRLR (compare the first and the second pair of bars in Fig. 20). A7 cells had a larger ruffling index than M2 cells (second pair of bars), which suggests that FLNa is sufficient, though not necessary, for PRL-dependent ruffling. As anticipated, WT SH2B1β enhanced PRL-induced membrane ruffling in A7 cells, compared to vector-expressing cells but did not enhance ruffling in M2 cells (black bars for vector and WT SH2B1β expressing cells in the presence of PRL). The WT SH2B1β-expressing A7 cells had a significantly higher ruffling index than that for WT SH2B1β-expressing M2 cells, suggesting that both FLNa and SH2B1β are needed for optimum PRL-induced membrane ruffling. Overexpression of SH2B1β Δ-Δ inhibited membrane ruffling in A7 cells but not M2 cells, indicating that the actin-binding domains of SH2B1β participate in actin rearrangement only when FLNa is present.

PAK1, a serine-threonine kinase, phosphorylates FLNa at serine 2152 which is required for PAK1-regulated membrane ruffling (Vadlamudi, Li et al. 2002). Previously, we demonstrated that JAK2 phosphorylates PAK1 on tyrosines 153, 201 and 285 upon PRL administration and that mutation of these three residues to phenylalanine results in a
Figure 19. JAK2 is tyrosyl phosphorylated in M2 and A7 cells overexpressing PRLR in response to prolactin. hPRLR was overexpressed in M2 and A7 cells. The cells were deprived of serum and treated with or without 400 ng/ml PRL for 15 min. JAK2 was immunoprecipitated with αJAK2. The immunoprecipitates and whole cell lysates were immunoblotted with indicated antibodies. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
Figure 20. Actin-binding domains of SH2B1β and Filamin A are required for maximal PRL-induced membrane ruffling. (A) The FLNa-deficient human melanoma M2 cell line and its derivative cell line (A7), which stably expresses FLNa were co-transfected with GFP-PRLR and myc-tagged WT SH2B1β or SH2B1β Δ-Δ mutant. Cells were serum-deprived and treated with 400 ng/ml PRL for 15 min. Filamentous actin was visualized by staining with AlexaFluor350-phalloidin and the transfected cells were visualized by both GFP fluorescence for PRLR and staining with amyc for SH2B1β. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 20 μm. (B) Ruffling index as a number of ruffles per cell was counted. White bars represent A7 cells and black bars represent M2 cells. Bars represent mean +/- S.E. *, p<0.05. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
reduction in cell motility (Rider, Shatrova et al. 2007). In this study we wanted to examine if PRL-dependent membrane ruffling is modulated by FLNa and pTyr-PAK1. We found that WT PAK1 overexpression in A7 cells led to the greatest amount of PRL-mediated membrane ruffling when compared to A7 cells overexpressing PAK1 Y3F (mutant of PAK1 that cannot be phosphorylated by JAK2) and M2 cells overexpressing WT PAK1, and PAK1 Y3F overexpression in M2 cells led to a reduction in membrane ruffling (Fig. 21). Our findings indicate that both FLNa and pTyr-PAK1 play a role in PRL-induced membrane ruffling.

To assess the role of SH2B1β, pTyr-PAK1 and FLNa in PRL-dependent cytoskeletal remodeling, we overexpressed GFP-PRLR, myc WT PAK1 or PAK1 Y3F, together with mRFP WT SH2B1β or SH2B1β Δ-Δ in M2 and A7 cells, treated the cells with PRL and calculated the ruffling index (Fig. 22). We determined that A7 cells overexpressing WT PAK1 and WT SH2B1β exhibited the highest PRL-dependent ruffling index, while the lack of JAK2 tyrosyl phosphorylation of PAK1 in PAK1 Y3F, the actin-binding ability of SH2B1β in the SH2B1β Δ-Δ mutant or the presence of FLNa in M2 cells led to a reduction in PRL-induced cell ruffling. In addition, and perhaps most crucial, overexpression of PAK1 Y3F and SH2B1β Δ-Δ in A7 cells still allowed for a response to PRL with membrane ruffling, while M2 cells overexpressing the same mutants were unable to respond to PRL (two last pairs of bars in Fig.22), suggesting that the presence of FLNa, WT SH2B1β and pTyr-PAK1 are necessary for maximal PRL-dependent membrane ruffling.

In order to determine how SH2B1β might remodel the actin cytoskeleton in a FLNa-dependent way, we decided to identify the nature of the relationship between
Figure 21. Tyrosyl phosphorylated PAK1 and Filamin A are required for maximal PRL-induced membrane ruffling. (A) M2 and A7 cell lines were co-transfected with GFP-PRLR and myc-tagged WT PAK1 or Y3F PAK1 mutant. Cells were treated as in Fig. 3. Filamentous actin was visualized by staining with AlexaFluor350-phalloidin and the transfected cells were visualized by both GFP fluorescence for PRLR and staining with αmyc for PAK1. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 20 μm. (B) Ruffling index as a number of ruffles per cell was counted. White bars represent A7 cells and black bars represent M2 cells. Bars represent mean +/- S.E. *, p<0.05. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
Figure 22. The actin-binding domains of SH2B1β, pTyr-PAK1 and Filamin A are required for maximal PRL-induced membrane ruffling. (A) A7 and M2 cell lines were co-transfected with GFP-PRLR, mRFP-tagged WT SH2B1β or SH2B1β Δ-Δ mutant, and myc-tagged WT PAK1 or PAK1 Y3F mutant. Cells were treated as in Fig. 3. Myc-PAK1 was visualized by αmyc, while PRLR was visualized by GFP fluorescence, and mRFP fluorescence for SH2B1β. PAK1 WT and/or SH2B1β WT or Δ-Δ localization in ruffles was used to count total number of ruffles per transfected cell. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 20 μm. (B) Ruffling index as a number of ruffles per cell was counted. White bars represent A7 cells and black bars represent M2 cells. Bars represent mean +/- S.E. *, p<0.05. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
FLNa and SH2B1β. We initially performed a co-immunoprecipitation experiment by immunoprecipitating endogenous SH2B1β and immunoblotting with antibody to FLNa, demonstrating that endogenous FLNa associates with endogenous SH2B1β (Fig. 23).

To elucidate whether SH2B1β and FLNa directly bind to each other, we utilized GST-tagged truncation mutants of FLNa: FLNa-1 (FLNa actin-binding domain; amino acids 1-278), FLNa-2 (FLNa repeats 1–10; amino acids 279-1252), FLNa-3 (FLNa repeats 11–16; amino acids 1253-1862), FLNa-4 (repeats 17–23 and dimerization domain; amino acids 1863-2647); FLNa-5 (dimerization domain; amino acids 2523-2647). These proteins are schematically depicted in Figure 24. Myc-tagged WT SH2B1β was in vitro translated and incubated either with GST or various GST-tagged truncation constructs of FLNa. SH2B1β was able to bind directly to FLNa-4 mutant (repeats 17-23 and dimerization domain) but not the other mutants, suggesting that repeats 17-23 of FLNa contain the binding site for SH2B1β (Fig. 24).

To identify the FLNa-binding site in SH2B1β, we performed in vitro binding assays in which GST or GST FLNa-4 was incubated with various in vitro-translated myc-tagged truncation mutants of SH2B1β. The SH2B1β mutant containing only amino acids 1-260 was able to bind directly to GST FLNa-4, while the SH2B1β mutants containing amino acids 1-105 and 1-200 were unable to bind, suggesting that amino acids 200-260 of SH2B1β are the FLNa-binding domain (Fig. 25). In order to verify that amino acids 200-260 of SH2B1β are the site for direct binding to FLNa, we constructed a mRFP SH2B1β mutant containing only amino acids 200-260 which we in vitro translated and tested for its ability to bind GST or GST FLNa-4. Consistent with our previous experiments, mRFP SH2B1β 200-260 bound directly to GST FLNa-4 but not GST (Fig.
Figure 23  Endogenous SH2B1β associates with endogenous Filamin A. Lysate of 293T cells (lane 3) was incubated either with αSH2B1β (lane 1) or with IgG (lane 2). Nitrocellulose was incubated with αFLNa. The same blot was reprobed with αSH2B1β for SH2B1β detection. L. Rider and M. Diakonova, *Mol. Endocrinology*, in press
**Figure 24. FilaminA repeats 17-23 is the site for SH2B1β interaction.** (A) Schematic depiction of FLNa truncations (modified from Cukier et. al., 2007). (B) Different GST-tagged truncated FLNa mutants were incubated with *in vitro* translated myc-SH2B1β. Bound myc-SH2B1β was detected by IB with αmyc. Lane 1 – GST; lane 2 – FLNa-1 (actin-binding domain of FLNa); lane 3 – FLNa-2 (repeats 1-10); lane 4 – FLNa-3 (repeats 11-16); lane 5 – FLNa-4 (repeats 17-23 and dimerization domain), lane 6 – FLNa-5 (dimerization domain). L. Rider and M. Diakonova, *Mol. Endocrinology*, in press.
Figure 25. Amino acids 200-260 of SH2B1β is the site for FLNa interaction. (A) Different *in vitro* translated myc-tagged truncated SH2B1β mutants were incubated with either GST (lanes 1, 3 and 5) or GST-tagged FLNa-4 (lanes 2, 4 and 6). Bound myc-SH2Bβ mutant was detected by IB with αmyc. Lanes 1 and 2 – amino acids 1-105 of SH2B1β; lanes 3 and 4 – amino acids 1-200 of SH2B1β; lanes 5 and 6 – amino acids 1-260 of SH2B1β. (B) mRFP-tagged (200-260) amino acids mutant of SH2B1β was translated *in vitro* in the presence of $^{35}$S methionine and incubated with either GST (lane 1) or GST-tagged FLNa-4 (lane 2). Bound mRFP-SH2Bβ mutant was detected by autoradiogram (48 h of film exposure). Equal amounts of $^{35}$S methionine-labeled SH2B1β input is shown on the right (24 h of film exposure). Amount of GST or GST-FLNa-4 was detected by IB with αGST. L. Rider and M. Diakonova, *Mol. Endocrinology*, in press.
In summary, our data show that SH2B1β is a novel FLNa-binding protein and amino acids 200-260 of SH2B1β are the site of direct binding to repeats FLNa-4 (repeats 17-23, amino acids 1863-2522).

Previously, we had demonstrated that both WT SH2B1β and the Δ-Δ mutant localize to membrane ruffles in 3T3 F442A cells (Rider, Tao et al. 2009). To determine the role of the FLNa-binding domain in SH2B1β localization, we expressed mRFP-tagged WT SH2B1β or 3Δ (SH2B1β mutant lacking both actin-binding domains and the FLNa-binding domain) along with GFP-PRLR in M2 or A7 cells. Both WT SH2B1β and 3Δ mutant co-localize with actin in cell ruffles in A7 and M2 cells (Fig. 26). Also intriguing was our observation that M2 cells not only ruffled but displayed bleb formation as mentioned in a previous study (Flanagan, Chou et al. 2001). To assess the relationship between SH2B1β and FLNa binding in PRL-mediated cytoskeletal remodeling, we co-expressed GFP-PRLR, myc-tagged PAK1 WT or PAK1 Y3F, and mRFP-tagged SH2B1β WT or SH2B1β 3Δ in M2 and A7 cells (Fig. 27). Texas Red-phalloidin was used to stain M2 and A7 cells transfected with GFP-PRLR only. M2 and A7 cells expressing GFP-PRLR, myc-tagged PAK WT or Y3F and mRFP-tagged SH2B1β WT or 3Δ mutant were observed by fluorescence microscopy and the ruffling index was determined by counting the total number of ruffles for 100 cells for each experimental condition using mRFP-SH2B1β and/or myc-PAK1 as a ruffle marker, since both proteins localize to membrane ruffles. As anticipated, expression of WT PAK1 and WT SH2B1β in A7 cells led to the maximum amount of PRL-dependent membrane ruffling, whereas expression of PAK1 Y3F and SH2B1β 3Δ in both cell lines abolished the PRL-stimulated ruffling response. These data suggest that pTyr-PAK1 and SH2B1β
Figure 26. SH2B1β WT and SH2B1β 3Δ co-localize with actin in the ruffles of A7 and M2 cells. M2 and A7 cells overexpressing mRFP-tagged SH2B1β WT or 3Δ mutant were stained with phalloidin-AlexaFluor 647 for actin visualization. Arrows indicate ruffles and asterisks denote blebs. Scale bar, 20 μm. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
Figure 27. The Filamin A-binding domain of SH2B1β, pTyr-PAK1 and Filamin A are required for PRL-induced membrane ruffling. (A) A7 and M2 cell lines were co-transfected with GFP-PRLR, mRFP-tagged WT SH2B1β (not shown) or SH2B1β 3Δ mutant (shown), and myc-tagged WT PAK1 (not shown) or PAK1 Y3F mutant (shown). Cells were treated as in Fig. 3. Myc-PAK1 was visualized by αmyc, while PRLR was visualized by GFP fluorescence, and mRFP fluorescence for SH2B1β. PAK1 and SH2B1β localization in ruffles was used to count total number of ruffles per transfected cell. Arrows indicate ruffles and asterisks denote transfected cells. Scale bar, 20 μm. (B) Ruffling index as a number of ruffles per cell was counted. White bars represent A7 cells and black bars represent M2 cells. Bars represent mean +/- S.E. *, p<0.05. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
work in complex with FLNa downstream of activated JAK2 in order to mediate PRL-dependent cytoskeletal remodeling. Previously, M2 and A7 cell migration was measured using the Transwell migration assay or by wounding assay (Klaile, Muller et al. 2005; Nishita, Yoo et al. 2006; Nomachi, Nishita et al. 2008). To further elucidate how pTyr-PAK1 and the FLNa-binding activity of SH2B1β might contribute to PRL-induced cell motility, we performed a wounding assay. Wound closure of M2 and A7 cells expressing GFP-PRLR, myc-tagged PAK1 WT or PAK1 Y3F, and mRFP-tagged SH2B1β WT or SH2B1β 3Δ was measured after 24 hr of cell deprivation, followed by wounding in the presence or absence of PRL. The percentage wound closure in 8 hr was determined and graphed for each condition (Fig. 28). Expression of WT PAK1 and WT SH2B1β in A7 cells led to the greatest PRL-dependent migration of cells into the wound, while expression of both PAK1 Y3F and SH2B1β 3Δ inhibited cell migration in response to PRL, demonstrating that PRL-mediated cell migration is regulated via PRL activation of JAK2 and formation of a complex containing FLNa, pTyr-PAK1 and SH2B1β.

In order to determine how the SH2B1β-PAK1-FLNa complex might work in modulating motility of breast cancer cells, we used T47D cell clones stably expressing GFP, GFP-containing PAK1 WT or PAK1 Y3F. We transiently overexpressed mRFP, mRFP-tagged SH2B1β WT or SH2B1β 3Δ in these stable cell lines and assessed wound closure as described above (Fig. 28). Expression of WT SH2B1β in WT PAK1 T47D stable cell lines led to a greater migration of cells into the wound in response to PRL than T47D GFP stable cells expressing mRFP. In comparison, expression of SH2B1β 3Δ in PAK1 Y3F T47D stable cell lines led to an inhibition of wound closure in response to
Figure 28. The Filamin A-binding domains of SH2B1β, pTyr-PAK1 and Filamin A are required for PRL-induced cell migration. (A) A7 and M2 cell lines were cotransfected with GFP-PRLR, mRFP-tagged WT SH2B1β or SH2B1β 3Δ mutant, and myc-tagged WT PAK1 or PAK1 Y3F mutant. Following deprivation, monolayers of cells were scarified and incubated in deprivation media with or without 400 ng/ml PRL. The percentage wound closure in 8h was calculated for each condition. Bar represent mean +/− S.E. *, p<0.05. (B) Clones of T47D cells stably expressing GFP, GFP-containing PAK1 WT or PAK1 Y3F were transiently transfected with vector, WT SH2B1β or SH2B1β 3Δ mutant. Monolayers of cells were wounded in the presence or absence of 200 ng/ml PRL and assessed after 18 hr. Bars represent mean +/− S.E. *, p<0.05 compared with cells expressing vectors and treated with PRL. L. Rider and M. Diakonova, Mol. Endocrinology, in press.
PRL, demonstrating that PAK1, SH2B1β and FLNa work together to modulate PRL-induced migration of breast cancer cells.

**Discussion**

Prolactin works as both a hormone and cytokine. The PRLR, which mediates the effects of PRL, is a part of the cytokine receptor superfamily (Bazan 1990). The PRLR family contains several different forms, including the long (85-90 kDa), intermediate (65 KDa), and short (42 kDa) forms. Binding of PRL to the PRLR causes the activation of JAK and subsequent phosphorylation of Stat5, Stat3 and Stat1 (Campbell, Argetsinger et al. 1994; Goffin, Struman et al. 1994; Gouilleux, Wakao et al. 1994; Lebrun, Ali et al. 1994; Rui, Lebrun et al. 1994; DaSilva, Rui et al. 1996; Schaber, Fang et al. 1998; Yu-Lee, Luo et al. 1998). Mammary epithelial cells contain several other pathways that are activated by PRL, including the Ras-MAPKs (Das and Vonderhaar 1996; Das and Vonderhaar 1996; Schaber, Fang et al. 1998), protein kinase C (Waters and Rillema 1989; Banerjee and Vonderhaar 1992), Src family kinases and PI3K (Rane and Reddy 2000). PRL also enhances cell motility, contributing to metastasis of cancer (Clevenger, Furth et al. 2003).

PRL has been implicated in the development of human breast cancer. PRL increases the motility and invasiveness of breast cancer cells (Maus, Reilly et al. 1999; Gutzman, Rugowski et al. 2007). Together with these findings and studies in animals demonstrating an increase in metastasis with PRL, these data suggest that PRL plays an important role in metastasis and progression of tumors. The exact means by which PRL is able to modulate cytoskeletal remodeling has not yet been fully elucidated. One way
PRL might exert its effects on the cytoskeleton is through the activation of the serine-threonine kinase Nek3, activation of Rac and subsequent paxillin phosphorylation (Miller, DeMaria et al. 2005; Miller, Antico et al. 2007), though considering the complexity of cell migration, this is perhaps one of many possible different signaling pathways that might orchestrate PRL-dependent cell migration. The results discussed here provide evidence for a novel mechanism of PRL-dependent actin cytoskeletal rearrangement and cell motility via JAK2, FLNa, PAK1 and SH2B1β.

SH2B1β was first identified as a binding partner and substrate of JAK2 (Rui, Mathews et al. 1997), for review (Maures, Kurzer et al. 2007). JAK2 tyrosyl phosphorylates SH2B1β on tyrosines 439 and 494 (O’Brien, Argetsinger et al. 2003) and enhances PRL- (Rider, Shatrova et al. 2007), growth hormone- (Rui, Mathews et al. 1997; Rui and Carter-Su 1999; Nishi, Werner et al. 2005), and leptin-dependent activation of JAK2 (Li, Zhou et al. 2007). SH2B1β has already been demonstrated to play a significant role in actin rearrangement (Herrington, Diakonova et al. 2000; Diakonova, Gunter et al. 2002; O’Brien, Argetsinger et al. 2003; Diakonova, Helfer et al. 2007), and we have recently shown that SH2B1β binds and cross-links actin filaments through both of its two actin-binding domains, which are necessary for optimal PRL-mediated cell membrane ruffling (Rider, Tao et al. 2009). Many different types of cells display cell ruffling in response to different extracellular stimuli. Moving cells also display cellular membrane ruffles, which are thought to be necessary for directional cell migration. Therefore, membrane ruffling is thought to be an indication of enhanced response to extracellular stimuli and increased cell migration (Ridley 1994; Borm, Requardt et al. 2005). We hypothesized that SH2B1β could work with FLNa to regulate
the actin cytoskeleton downstream of PRL-activated JAK2, since another SH2B family member SH2B3 (Lnk) was previously shown to bind FLNa (He, Li et al. 2000). In order to examine our hypothesis, we expressed the PRLR in FLNa-deficient M2 cells and A7 cells, which are the FLNa-expressing derivative line of M2 cells. PRL was able to stimulate membrane ruffling in both lines, but WT SH2B1β significantly increased this effect in only A7 cells. SH2B1β has been previously shown to enhance growth hormone-, PDGF- (Herrington, Diakonova et al. 2000), and PRL-dependent cell ruffling (Rider, Tao et al. 2009), and the data we present here suggest that SH2B1β exerts these effects in a FLNa-dependent manner.

We showed that WT SH2B1β is able to amplify cell membrane ruffling in A7 cells, though not M2, suggesting that FLNa works with SH2B1β to regulate the actin cytoskeleton. We demonstrated that deleting both actin-binding sites in SH2B1β caused SH2B1β to act as a dominant negative when expressed in A7 cells (last white bar in Fig. 20), which supports our previous data with PRL administration in T47D, and indicates that SH2B1β plays a role in PRL-induced cell ruffling. However, we found that deletion of both actin-binding domains in SH2B1β did not inhibit PRL-dependent ruffling in M2 cells (last black bar in Fig. 20) implying that a pathway independent of FLNa is still operating, but that FLNa does indeed participate in SH2B1β-dependent regulation of the actin cytoskeleton. Taken together, we hypothesize that upon PRL binding to receptor, SH2B1β relocates to active receptor-JAK2 complexes, where it binds and cross-links actin filaments, mediating cytokine-dependent ruffling in a FLNa-dependent mode. Therefore, we propose that the dominant negative SH2B1β Δ-Δ may still be able to bind
JAK2 and FLNa, keeping both proteins from binding to endogenous SH2B1β, but is not able to crosslink actin filaments for membrane ruffling.

We have verified our previous data that the actin-binding domains of SH2B1β play a role in PRL-dependent membrane ruffling. Both M2 and A7 cell lines were able to respond to PRL when SH2B1β Δ-Δ was expressed, though to a significantly lesser extent than when WT SH2B1β was expressed (compare two last pairs of bars in Fig. 20). This suggested that the actin cytoskeleton might be regulated via an additional mechanism. We decided to test the possibility that the serine-threonine kinase PAK1 might be participating in this process for two reasons. We demonstrated that JAK2 phosphorylates PAK1 on tyrosines 153, 201 and 285 (Rider, Shatrova et al. 2007). In addition, PAK1 phosphorylates FLNa on serine 2152 and FLNa subsequently enhances the kinase activity and autophosphorylation of PAK1 (Vadlamudi, Li et al. 2002). In Figure 21 we have demonstrated WT PAK1 stimulates PRL-induced ruffling and that this process is not dependent on FLNa, as the ruffling index for both M2 and A7 was significantly higher when WT PAK1 was expressed. There have been similar findings in M2 cells treated with serum, which produced cellular membrane ruffles in a matter of minutes, but returned to blebbing hours later (Flanagan, Chou et al. 2001). EGF was also able to induce ruffle formation in M2 cells minutes after treatment (Fiori, Zhu et al. 2009). Cell ruffling has also been observed in M2 cells stimulated or not with various ligands (Lin, Canfield et al. 2002; Meng, Yuan et al. 2004; Park, Kim et al. 2005; Kim, Ridgway et al. 2007; Beekman, van der Poel et al. 2008; Byfield, Wen et al. 2009; Kasza, Nakamura et al. 2009; Sverdlov, Shinin et al. 2009; Li, Yu et al. 2010). However, heregulin- and sphingosine-treated M2 cells do not produce membrane ruffles, though
both ligands activate PAK1 (Vadlamudi, Li et al. 2002). It is likely that different actin-regulating pathways are activated downstream of heregulin, sphingosine and PRL. JAK2 phosphorylation of PAK1 is crucial for ruffling induced by PRL, since both M2 and A7 cells expressing PAK1 Y3F showed no amplification of cell ruffling (last two bars in Fig. 21). This indicates that PAK1 can modulate actin remodeling not only through phosphorylation of substrates, but possibly via protein-protein interactions with phosphorylated tyrosines 153, 201 and 285 of PAK1. We are working on identifying which actin-regulating proteins work together with pTyr-PAK1. Though PAK1 Y3F did not amplify ruffling like WT PAK1 (Fig. 21), it acted as a dominant negative inhibitor of PRL ruffling when co-expressed with SH2B1β Δ-Δ (last two bars in Fig. 22). It is possible that SH2B1β Δ-Δ binding to and sequestering potential PAK1 Y3F targets might have a more drastic effect when both SH2B1β and PAK1 are mutated. One possible candidate is Rac1, as SH2B1β associates with Rac1 and amino acids 85-106 of SH2B1β (which are still present in the SH2B1β Δ-Δ mutant) are required for this association (Diakonova, Gunter et al. 2002). Rac1 is also a well-established activator of PAK1. Another potential candidate is Grb2, as it binds both SH2B1β (Qian, Riccio et al. 1998) and PAK1 (Puto, Pestonjamasp et al. 2003). SH2B1β Δ-Δ might bind Rac1, Grb2 and/or other proteins and prevent them from associating with PAK1 Y3F and endogenous PAK1, impeding the formation of signaling complexes at the cell membrane needed for actin rearrangement.

We have also demonstrated that SH2B1β binds directly to FLNa through amino acids 200-260 of SH2B1β and repeats 17-23 of FLNa and examined the effects of a mutant of SH2B1β lacking both actin-binding domains and the FLNa-binding domain
(SH2B1β 3Δ). SH2B1β 3Δ eliminated PRL-induced membrane ruffling in M2 and A7 when co-expressed with PAK1 Y3F (last two pairs of bars in Fig. 27). SH2B1β Δ-Δ is still able to elicit membrane ruffling A7 cells when co-expressed with PAK1 Y3F (last two white bars in Fig. 27), however SH2B1β 3Δ prevents ruffling (last two white bars in Fig. 27), possibly because SH2B1β Δ-Δ binds FLNa still and is able to maintain a somewhat functional JAK2-SH2B1β Δ-Δ-FLNa-PAK1 Y3F-actin complex. However once FLNa and SH2B1β can no longer bind and SH2B1β no longer bind actin, as in the case of SH2B1β 3Δ, this leads to the destruction of a functional complex and prevents PRL signaling to the actin cytoskeleton. Disruption of the JAK2- SH2B1β-PAK1- FLNa complex eliminated both membrane ruffling and PRL-mediated wound closure (Fig. 28).

We have constructed a working model of PRL-mediated actin cytoskeletal remodeling that incorporates our current work with previous findings (Fig. 29). Once PRL binds to its receptor, JAK2 becomes activated, followed by recruitment of SH2B1β to the activated PRLR-JAK2 complexes, where it binds and cross-links actin, in addition to binding FLNa. FLNa then enhances the activation of PAK1, binds to SH2B1β bringing additional SH2B1β molecules to the JAK2-PAK1-FLNa complex. As SH2B1β enhances the activation of JAK2 (Rui and Carter-Su 1999), the formation of this complex leads to the augmentation of JAK2 activation and additional activation of the JAK2-PAK1-FLNa-actin complex, resulting in actin cytoskeletal remodeling. JAK2 tyrosyl phosphorylation of PAK1 and SH2B1β might also provide additional docking sites for SH2-domain containing actin regulating proteins relocating them to the membrane, mediating actin remodeling and ruffling. In support of this, it has previously been demonstrated that tyrosyl phosphorylation of SH2B1β tyrosines 439 and 494 by JAK2 is
Figure 29. Prolactin-activated JAK2 promotes formation of multiprotein complex. Schematic representation of the proposed working model. PRL-activation of JAK2 leads to tyrosyl phosphorylation of PAK1 on tyrosines 153, 201 and 285, thereby increasing PAK1 activities (both the serine/threonine kinase activity and ability to create potential protein-protein interactions) and stimulating phosphorylation of FLNa. Phosphorylated FLNa stimulates the kinase activity of PAK1 and has increased actin-regulating activity. FLNa which directly binds to SH2B1β, relocates SH2B1β to the JAK2-PAK1-FLNa complex. Since SH2B1β is the enhancer of the kinase activity of JAK2, the formation of the complex results in enhancement of JAK2 activation and further activation of the JAK2-PAK1-FLNa complex that leads to actin cytoskeleton reorganization via actin-regulating proteins PAK1, FLNa and SH2B1β. (Binding of FLNa to the plasma membrane is not shown). L. Rider and M. Diakonova, Mol. Endocrinology, in press
important for growth hormone-induced ruffling (O'Brien, Argetsinger et al. 2003). Also, Grb2 and/or other SH2 domain-containing proteins (such as actin-binding proteins cortactin, spectrin and myosin I) could potentially bind to the proline-rich region of SH2B1β and mediate PRL-dependent membrane ruffling. In summary, as PRL has been demonstrated to act as a chemoattractant for human breast carcinoma (Maus, Reilly et al. 1999), our findings might provide an additional mechanism of PRL-dependent metastasis of breast cancer.
V. Summary

In our studies we sought to determine how PRL activation of JAK2 leads to the remodeling of the actin cytoskeleton. We have identified PAK1 as a novel substrate of JAK2, which is directly phosphorylated on tyrosines 153, 201 and 285 by JAK2. Tyrosyl phosphorylation of PAK1 at these sites leads to the enhancement of PRL-induced membrane ruffling, migration, wound closure and invasion of breast cancer cells, implicating the PRL-JAK2-PAK1 pathway in human breast cancer.

We have also determined that another JAK2 substrate, SH2B1β, participates directly in the modulation of the actin cytoskeleton via binding and cross-linking actin filaments. We have identified amino acids 150-200 and 615-670 of SH2B1β as actin-binding domains and shown that these domains are required for the actin cross-linking activity of SH2B1β. We have also determined that both actin-binding domains of SH2B1β are necessary for the maximal GH- and PRL-dependent membrane ruffling of cells as well as for the maximal phagokinesis of cells. We demonstrated that the N-terminal actin-binding domain of SH2B1β (amino acids 150-200) is needed for the proper intracellular localization of SH2B1β as well as the presence of VASP.

Lastly, we demonstrated that a PAK1 substrate, FLNa, also binds directly to SH2B1β through amino acids 200-260 of SH2B1β and repeats 17-23 of FLNa. We have shown that pTyr-PAK1, SH2B1β and FLNa participate together in the regulation of the actin cytoskeleton downstream of PRL activation of JAK2. Indeed, pTyr-PAK1, the actin- and FLNa-binding domains of SH2B1β and the presence of FLNa are all required for the maximal PRL-mediated membrane ruffling and wound closure of cells, including
breast cancer cells. These data demonstrate that PRL activation of JAK2 promotes the formation of a multiprotein complex consisting of JAK2, SH2B1β, PAK1 and FLNa, leading to the remodeling of the actin cytoskeleton, and that this complex could serve as a relevant target in the treatment of human breast cancer.
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