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Leslie Cook
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Entitled

Filamin A Associates with the JAK2/PAK1 Complex and the JAK2/SH2B1β Complex

By
Leslie Cook

Submitted as partial fulfillment of the requirements for

The Master of Science in Biology

Advisor: Dr. Maria Diakonova

College of Graduate Studies

The University of Toledo

December 2008
An Abstract of

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PAK1 is an important regulator of many cellular functions and has been implicated in various types of cancer. PAK1 is phosphorylated by the receptor associated tyrosine kinase JAK2 on tyrosines 153, 201, and 285; this activates PAK1 and this activity can be increased by the adaptor protein SH2B1β (Rider, et al., 2007). SH2B1β increases JAK2 activity, which in turn increases JAK2-dependent PAK1 phosphorylation. We have demonstrated that the JAK2-dependent activation of PAK1 increases cell motility in Human Mammary Epithelial (HME) cells. Here our study is focused on understanding the mechanism behind this increase in cell motility and we suggest one possible pathway through the PAK1 effector Filamin A. Filamin A is an actin binding protein that is phosphorylated by PAK1 on Serine 2152.
We have found that Filamin A S^{2152} phosphorylation is increased in the presence of JAK2-dependent phosphorylation of PAK1. SH2B1β does not increase the PAK1-dependent Filamin A phosphorylation as we would expect, but competes with PAK1 to bind to Filamin A. We have demonstrated for the first time that Filamin A is tyrosyl-phosphorylated in a JAK2-dependent manner. Finally, JAK2 tyrosyl-phosphorylation of Filamin A is increased in the presence of SH2B1β or PAK1. Together, these observations suggest that Filamin A either forms a complex with PAK1 and JAK2 or with SH2B1β and JAK2 which facilitates the Filamin A tyrosyl-phosphorylation by JAK2. The Filamin A, PAK1 and JAK2 complex also facilitates phosphorylation of Filamin A S^{2152} by PAK1.
Acknowledgements

I would first like to thank my advisor, Dr. Diakonova, for her guidance throughout my graduate work. She was willing to teach me a lot about research, as I had not had any research experience before starting this program. I was unsure about most things that were involved with pursuing a graduate degree when I first started, and she guided me into the lab where I began to feel like I belonged here.

Also, I would like to thank the members of my lab. They were always willing to discuss any problems that I was having and always offered their advice. Everyone was always willing to help when they could if I was unable to finish something on my own. Without the help from Leah, and her taking care of the everyday labors of lab, I don’t quite know how I would have gotten as much accomplished as I did. Xiaofeng was always finding quicker and easier ways to do stuff around the lab that helped all of us save precious time. Jing also helped me with experiments where she had already worked out the protocol, which saved me quite a bit of time. All of the members of the Chadee lab were great to bounce ideas off of since they did not always know everything we were doing in lab they could offer a fresh new perspective on many things. I also was able to explain things to them to see if I was making my point clear to someone who doesn’t know much about my research.

My family has been very supportive during my time in school. My husband, Joe, has been my support for the past two years while pursued a graduate degree. I would not have made it through this time without him. My parents were always willing to listen when I was concerned I wasn’t making any progress and my dad has always been interested in my research and we often discussed what I was working on.
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**Introduction**

Breast cancer is the second most common cancer in women and accounts for over 25 percent of women that are diagnosed with cancer in the United States (American Cancer Society, 2007). p21 activated kinase 1 (PAK1) has been implicated in cellular transformation in several types of cancer, including breast cancer. Both PAK1 expression and its kinase activity are increased in breast tumors (Balasenthil, et al., 2004). We are interested in the potential role of PAK1 in cell motility and tumorogenesis.

**PAK1**

PAK1 belongs to a family of serine-threonine kinases that are activated by both GTPase-dependent and -independent pathways that modulate cell motility, gene regulation, cell survival, and mitosis (Figure 1A). The PAK1 gene is amplified in ovarian and bladder cancers and protein expression is increased in breast, colon, ovarian, and brain cancers (for review, Kumar, et al., 2006). PAK1 is also necessary for Ras, Rac1, and Rac3 induced transformation in certain cell types (Tang, et al., 1997; Tang, et al., 1999; Mira, et al., 2000). Identifying downstream targets of PAK1 that are involved in changes in phenotypes that correspond to tumorogenesis could elucidate possible targets for cancer treatment.

PAK1 contains an N-terminal autoinhibitory dimerization domain (Figure 1B); the inhibitory domain of one subunit blocks the kinase domain of the other (Parrini, et al.,
Figure 1: Signaling through the serine/threonine kinase PAK1 involves many different regulators and targets. (A) PAKs are activated by Rho GTPases, adaptor proteins, and kinases. The PAK effectors include many different cellular targets that regulate most levels of cellular function. (B) Schematic of the PAK1 protein. PBD-p21 Binding Domain; AI-Auto Inhibitory region.
2002). The inhibitory domain is released when a small GTPase (Rac or CDC42) or other activator binds to the PAK1 p21 binding domain (Lei, et al., 2000). Once the autoinhibition of PAK1 is released, T423 can be phosphorylated preventing the autoinhibitory domain from re-binding to the kinase domain. PAK1 phosphorylates many substrates including adaptor proteins, kinases, and cytoskeletal proteins. The PAK1 N-terminal PXXP motif can associate with adaptor proteins that contain an SH3 domain, such as Nck or Grb2 (Bokoch, 2003). The binding of PAK1 to Nck relocalizes PAK1 to the plasma membrane and increases PAK1 kinase activity even in the absence of other PAK1 activators (Lu, et al., 1997). Another way to increase PAK1 kinase activity is by phosphorylation of T423 (Zenke, et al., 1999). Phosphorylation at this site mimicked by PAK1T423E, a constitutively active mutant, causes multiple mitotic spindles to form in single cells and increases anchorage-independent growth and directional cell motility (Vadlamudi, et al., 2000; Sells, et al., 1999). A kinase inactive mutant, PAK1K299R, reduces the invasiveness of MDA-MB231, a breast cancer cell line (Adam, et al., 2000). These phenotypic changes associated with constitutively active and kinase inactive PAK1 suggest that changes in PAK1 kinase activity alters the phosphorylation of downstream targets ultimately leading to the phenotypic changes associated with transformation.

**JAK2 Phosphorylates PAK1**

Another recently discovered mechanism of activation of PAK1 is JAK2-dependent phosphorylation on tyrosines 153, 201 and 285. Tyrosyl-phosphorylation of PAK1 protects cells from apoptosis and may also modulate other functions that are downstream of PAK1, such as cell motility, migration, and differentiation. For example,
mutation of the three previously mentioned tyrosines prevents PAK1 tyrosyl-phosphorylation and cells are no longer protected from apoptosis (Rider, et al., 2007). We are interested in how the JAK2-dependent phosphorylation of PAK1 modulates cell motility and how cell motility changes when PAK1 phosphorylation by JAK2 is inhibited.

JAK2 is a member of the receptor associated tyrosine kinase family and is involved in cytokine-mediated cellular responses, including the activation of transcription. JAK2 is activated by growth hormone, prolactin, leptin and other ligands that bind receptors belonging to the cytokine hematopoietin superfamily (for review, Ihle, et al., 1998). Upon receptor stimulation, JAK2 is recruited to the receptor, dimerizes, then trans-phosphorylates (for review, Hou, et al., 2002). Activated JAK2 phosphorylates the receptor, as well as downstream targets. One family of downstream targets that JAK tyrosine kinases activate are signal transducers and activators of transcription (STATS). STATS then relocate to the nucleus and activate specific genes. Since JAK2 is activated by growth factors and cytokines, PAK1 can also be activated by these factors. Together, these observations suggest that JAK2 is a potentially important regulator of PAK1 and targets that are downstream of PAK1.

**SH2B1β Enhances JAK2-dependent PAK1 Phosphorylation**

Another substrate of JAK2 is the adaptor protein SH2B1β (Rui, et al., 1997). The SH2B family includes SH2B1, with 4 isoforms (α, β, γ, and δ), SH2B2 (APS), and SH2B3 (Lnk). SH2B1β binds to JAK2 and increases JAK2 autophosphorylation and the JAK2-dependent phosphorylation of PAK1 (Rui et al., 1997; O’Brien, et al., 2002; Rider,
et al., 2007). The SH2 domain of SH2B1β binds to phosphorylated tyrosine 813 of JAK2 and JAK2 phosphorylates SH2B1β on tyrosines 439 and 494 (Kurzer, et al., 2004; O’Brien, et al., 2003). When these phosphorylation sites are mutated, SH2B1β-dependent membrane ruffling in response to growth hormone treatment is decreased (O’Brien, et al., 2003). SH2B1β is required for maximal cell motility, as well as wound migration, when cells are treated with growth hormone (Diakonova, et al., 2002). Since SH2B1β enhances ruffling (induced by growth hormone), which requires actin reorganization, we previously predicted that SH2B1β will either directly or indirectly be involved in the reorganization of actin.

**PAK1 Phosphorylates Filamin A**

Since JAK2 phosphorylation increases PAK1 kinase activity, we have tentatively identified several effectors of PAK1 that may be involved in phenotypic changes due to JAK2 phosphorylation of PAK1; one of these is the actin binding protein Filamin A. Filamins have long β-sheet repeats that give the protein a rigid structure (for review, Stossel, et al., 2001). Three human Filamins (A, B, and C) have been characterized; Filamin A, the most abundant form, homodimerizes and binds to F-actin (Shizuta, et al., 1976). Filamin A contains an actin-binding region at the N-terminus that is similar to the actin-binding sequence of α-actinin and spectrin, and 24 repeats of about 96 amino acids (Gorlin, et al., 1990). Dimerization of the Filamin A C-terminus leaves the N-terminus free to bind to actin. Filamin A arranges actin into loose, gel-like networks and is the strongest actin-crosslinking protein, requiring the lowest concentration of Filamin in order to form actin gels compared to other actin-gelling proteins (Brotschi, et al., 1978).
Figure 2: Structure of Filamin A. Filamin A dimerizes at the C-terminus (repeat 24) and contains an actin binding domain at the N-terminus. The remainder of the protein is composed of 24 β-sheet repeats (~96 aa) and two hinge regions.
Filamin A also binds to at least 20 other proteins, including integrins, receptors, Rho small GTPases, SH2B3 and potassium channels (for review, Stossel, et al., 2001), suggesting that Filamin A is involved in several signaling pathways that potentially regulate actin reorganization.

Filamin A is phosphorylated by several kinases. For example, phosphorylation by Protein Kinase A protects Filamin A from protease cleavage by calpain (Chen and Stracher, 1989). Phosphorylation by Ca\(^{2+}\)/calmodulin-dependent protein kinase II lowers the actin binding affinity of Filamin A and its ability to form actin gels (Ohta and Hartwig, 1995). Filamin A is also phosphorylated by Ribosomal S6 Kinase (Ohta and Hartwig, 1996). Ribosomal S6 Kinase inhibition by the MEK inhibitor UO125 reduces Filamin A phosphorylation, however, some residual Filamin A phosphorylation remains, demonstrating that Filamin A phosphorylation is regulated by several independent mechanisms (Woo, et al., 2004). Filamin A is also phosphorylated by PAK1 and is required for PAK1-dependent ruffle formation (Vadlamudi, et al., 2002). Filamin A binds to, and colocalizes with PAK1 in membrane ruffles when PAK1 is activated by heregulin or sphingolipids (Vadlamudi, et al., 2002). Filamin A repeat 23 binds to the PAK1 CRIB domain and PAK1 phosphorylates Filamin A on S\(^{2152}\) (Vadlamudi, et al., 2002). Since multiple kinases regulate Filamin A, this suggests that Filamin A is an important regulator of the actin cytoskeleton and may be involved in a variety of cellular responses.

Another binding partner of Filamin A is SH2B3. Filamin A binds to SH2B3 (LNK) via an interdomain region between the pleckstrin and Src homology (SH2) domains of SH2B3 (He, et al., 2000). This interdomain region is present in SH2B1\(\beta\)
Figure 3: The SH2B Family of Proteins. The SH2B family contains six members. SH2B1 which has four isoforms, α, β, γ, and δ, SH2B2 and SH2B3. The former names for SH2B2 and SH2B3 are listed in parentheses. DD-Dimerization Domain, P-Proline Rich Region, PH-Pleckstrin Homology Domain, SH2-Src Homology 2 Domain.
leading us to speculate that SH2B1β also associates with Filamin A. Since all but the C-terminus is conserved in the four SH2B1 isoforms, we would expect that if Filamin A can bind to SH2B1β it will also bind to the other 3 isoforms. If Filamin A and SH2B1β (or other isoforms) can associate with each other, this may be another mechanism used to regulate the actin cytoskeleton through Filamin A.

JAK2 phosphorylates PAK1 and this phosphorylation is enhanced by SH2B1β (Rider, et al., 2007). This phosphorylation and increase in PAK1 activity may alter many pathways downstream of PAK1. One possible downstream target of PAK1 that could be altered is Filamin A. Therefore, the present study was designed to examine the potential role of tyrosyl-phosphorylated PAK1 in modulating actin cytoskeletal dynamics and cell motility through the interaction of PAK1 with JAK2, SH2B1β, and Filamin A. We hypothesize that increasing PAK1 activity by JAK2-dependent phosphorylation of PAK1 will increase cell motility. Also, we hypothesize that the mechanism for the increase in cell motility will involve an increase in phosphorylation of PAK1 cytoskeletal substrates, including Filamin A, or an increase in binding of PAK1 to its cytoskeletal targets.
**Materials and Methods**

**Tissue Culture:**

Human Mammary Epithelial cells (from Drs. Ethier (Karmanos Cancer Institute, MI) and Band (Northwestern University, IL)) were grown in Ham’s F-12 Media (Cellgro) supplemented with 5% Fetal Bovine Serum (Hyclone), 5 µg/ml Insulin (Sigma), 1 µg/ml Hydrocortisone (Sigma), 10 ng/ml Epidermal Growth Factor (Sigma), 100 ng/ml Cholera Toxin (Sigma), 2.5 µg/ml Fungizone (Invitrogen), 5 µg/ml Gentamicin (Invitrogen), and 2.5 µg/ml Plasmocin (Amaxa). They were kept in an incubator with 10% CO$_2$ at 37°C. 293T and 3T3 F442A cells, from American Type Culture Collection (ATCC) and Dr. Green (Harvard Univeristy) respectively, were grown in Dulbecco’s Modified Eagle’s Medium (DMEM—Cellgro) supplemented with 10% calf serum (Cellgro), 1 mM L-Glutamine (Hyclone), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. These cells were kept in a 37°C incubator with 5% CO$_2$ (293T) or 10% CO$_2$ (3T3 F442A). Deprivation Media was made by supplementing DMEM with 1% BSA instead of 10% calf serum.

**Transfection:**

One confluent 10 cm tissue culture dish of cells was split to three dishes 16-24 hours prior to transfection. DNA (5-10 µg of DNA per dish) was added to 0.5 ml of 0.25M CaCl$_2$. Then 0.5 ml of 2X HBS (pH 6.95) was added dropwise to the tube while
vortexing. The tubes were incubated on ice for ten minutes. The contents were then added dropwise to the dish containing the cells that were plated the previous day. The dishes were returned to the incubator then four to six hours later they were washed twice with DMEM (no supplements) and then normal media was replaced. Approximately 30 hours after transfection, the dishes were washed twice with Phosphate Buffered Saline Dulbecco’s (DPBS) then deprivation media was added to the dishes.

**Colloid Gold Assay:**

Coverslips were coated with gold by first incubating them on 100 µg/ml polylysine for 15 minutes at room temperature. They were then washed three times for three minutes in Phosphate Buffered Saline (PBS). The coverslips were then incubated for 15 minutes in 1% BSA. They were again washed three times for three minutes in PBS. Next, the hot gold solution (11 ml H₂O, 1.8 ml AuCl₃, 6 ml Na₂CO₃, boiled then add 1.8 ml 0.1% formaldehyde) was incubated on the coverslips for 45 minutes. The coverslips were then washed in PBS and stored in PBS plus 0.01% azide.

250,000 to 300,000 HME cells were plated per well of a six well plate 16-24 hours prior to transfection. They were transfected following the Expressfect protocol. The cells were washed three hours after transfection and regular media was replaced. 30 hours post-transfection, cells were trypsinized and plated on the Colloid Gold coated coverslips. The cells were returned to the incubator and after 16 hours they were fixed using 4% paraformaldehyde for 30 minutes then washed for five minutes three times in PBS and then permeabilized for 15 minutes with 0.1% Triton X-100. The coverslips were then washed for five minutes three times in blocking buffer (2% goat serum in PBS) then
incubated with monoclonal anti-HA (1:100, Roche) antibody for 45 minutes. They were washed again three times for five minutes with blocking buffer and then incubated for 45 minutes with anti-mouse-FITC (1:200) and phalloidin-Texas Red (1:60). The coverslips were washed for five minutes a final three times then mounted onto microscope slides using 5% propyl gallat (in 90% glycerol).

Images were taken using phase contrast and a FITC filter set to visualize the area cleared of gold and transfected cells, respectively. The images were overlayed in Adobe Photoshop. Image Tool was used to determine the phagokinetic index. The area of the cell was measured by outlining the cell and the area that was cleared of gold was also measured. The area cleared of gold was divided by the area of the cell to determine the phagokinetic index. The mean phagokinetic index was calculated and plotted with standard error of the mean.

**Immunoprecipitations:**

48 hours after transfection the dishes were washed three times with PBSV (10 mM NaPO₄, 150 mM NaCl, 1 mM Na₃VO₄, pH 7.0). Lysis buffer was prepared by supplementing L-RIPA (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, pH 7.5) with 10 mM Na₃VO₄ (10 times standard protocol of 1 mM), 50 mM NaF, 1mM PMSF, 100 µg/ml aprotinin (10 times standard protocol of 10 µg/ml ), 100 µg/ml leupeptin (10 times standard protocol of 10 µg/ml ). 0.8 ml of lysis buffer was added to each dish and the cell lysates were scraped off the dish and put in a microcentrifuge tube. The cell lysates were incubated on ice for ten minutes and then centrifuged at 13,000 g at 4°C for ten minutes. 50 µl of the cell lysate was placed in a new microcentrifuge tube
along with 12.5 µl of 4X Laemmli sample buffer (LSB). The remainder of the cell lysate was pipetted into a new microcentrifuge tube and were incubated for two hours at 4°C with a 1:100 dilution of monoclonal anti-myc (University of Michigan Hybridoma Core Facility) or anti-SH2B1β. Then 30 µl of protein A beads was added and rotated at 4°C for either one or five hours. The protein A beads were pelleted by centrifugation at 13,000 g and were washed three times in lysis buffer. 70 µl of LSB sample buffer was added to the beads and they were boiled for five minutes. The samples were run on an 8% acrylamide gel at 10 mA overnight.

**Western Blot**

The proteins from the gel were transferred to PVDF membrane at 100 V for one and a half hours. The membrane was incubated for four hours in 3% BSA blocking buffer at 4°C. Then the indicated primary antibody (monoclonal anti-HA, Covance; polyclonal anti-Filamin A and anti-phospho-Filamin A, Cell Signaling; anti myc and anti-SH2B1 same as for immunoprecipitation) was added to the membrane to incubate overnight. The membrane was washed for ten minutes three times in Tris Buffered Saline (TBS)-Tween and rinsed in TBS. Then secondary antibody (goat anti-mouse-HRP, 1:7500, or Protein-A-HRP, 1:5000, diluted in TBS) was added to the membrane and incubated for one hour at 4°C. The membrane was then washed three more times in TBS-Tween. After rinsing the membrane in TBS the membrane was incubated in a chemiluminescent solution for five minutes before exposing to film to visualize the proteins.
**In vitro Translation**

Filamin A-myc was translated *in vitro* using the TnT® T7 Coupled Reticulocyte Lysate System (Promega). The protocol was followed using \[^{35}\text{S}]\text{methionine. After the translation reaction was complete, 2 µl of the translation product was added to 20 µl LSB and boiled for two minutes. This sample was run on an 8\% acrylamide gel and then transferred for PVDF membrane. The membrane was exposed to film overnight to detect the }^{35}\text{S incorporated into the translation product.}

**In vitro Binding**

Affinity purified GST and SH2B1\(\beta\)-GST proteins were eluted from the glutathione agarose beads and run on a gel. The gel was transferred and blotted with anti-GST to determine the relative amount of protein attached to the beads. The amount of GST and SH2B1\(\beta\)-GST beads used for the binding experiment was adjusted to have equal amounts of GST and SH2B1\(\beta\)-GST. Unbound glutathione agarose beads were added as necessary to have an equal volume of beads for each tube. *In vitro* translated \[^{35}\text{S}]\text{Filamin A-myc (50 µl) was rotated with 30 µl of purified GST or SH2B1\(\beta\)-GST attached to glutathione beads at 4\°C for two hours in 200 µl of binding buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 30 mM MgCl}_2, 40 mM NaCl, 0.5\% Nonidet P-40 Alternative). The beads were then washed three times in the binding buffer and two times in binding buffer without 0.5\% Nonident P-40 Alternative. Then 60 µl of LSB was added to the beads and they were boiled for five minutes. The samples were run on an 8-16\% gradient gel (Biorad) then transferred to PVDF membrane. The membrane was exposed
to film overnight to detect Filamin A-myc. The membrane was then probed for anti-GST and anti-myc using the western blot procedure listed above.
Results

**JAK2-dependent Phosphorylation of PAK1 Increases Cell Motility**

To determine if tyrosyl phosphorylation of PAK1 by JAK2 has an effect on cell motility, we performed a phagokinetic assay. Human Mammary Epithelial (HME) cells were plated on coverslips that were coated with colloid gold. As the cells phagocytosed the gold particles, they cleared a path showing where they had moved through the gold. The area cleared was measured and divided by the area of the cell; this number is called the phagokinetic index and is plotted. We found that HME cells transfected with wild type PAK1 and wild type JAK2 or constitutively active mutant PAK1 (PAK1_{T423E}) had a higher phagokinetic index and therefore cell motility than those transfected with wild type PAK1 and kinase-inactive JAK2 (Figure 4). We also used a PAK1 mutant (Y3F) that has the three tyrosines that are phosphorylated by JAK2 mutated to phenylalanine so that it cannot be phosphorylated by JAK2. We also observed that cells transfected with wild type JAK2 and wild type PAK1 had greater cell motility than cells transfected with PAK1 Y3F. The phagokinetic index of untransfected cells was higher, but not statistically different, than cells transfected with wild type JAK2 and wild type PAK1 and those transfected with PAK1_{T423E}. We attribute the decrease in cell motility for all transfected cells to the toxicity of the Expressfect reagent that was used to transflect the cells. We tried several different methods of transfection and Expressfect was the most efficient, but even with the minimum incubation of reagent with the cells it still killed some of the
Figure 4: Inhibition of JAK2-dependent phosphorylation of PAK1 decreases cell motility in HME cells. HME cells transfected with the indicated forms of PAK1 and JAK2 were plated on colloid gold-coated coverslips. Images of transfected cells were captured using a fluorescein isothiocyanate filter set (B and D stained with anti-HA for PAK1) and differential interference contrast (A and C). The area cleared of colloid gold (outlined) and the area of the cell was measured. Scale bar, 20 µm. Phagokinetic index was calculated by dividing the area cleared of gold by the area of the cell (E). Mean phagokinetic index ± S.E. was plotted, *p<0.05 compared to WT PAK1 and WT JAK2.
cells. Based on these data, we conclude that this increase in cell motility is one downstream effect of the JAK2-dependent tyrosyl phosphorylation of PAK1. The mechanism for this increase in motility is still unclear.

In order to begin to understand this mechanism, we examined several known PAK1 targets that participate in cell motility to determine which were regulated by JAK2-dependent phosphorylation of PAK1 (Figure 5). We decided to focus on Filamin A as our target of interest.

**JAK2-dependent Phosphorylation of PAK1 Increases Filamin A Phosphorylation**

Since Filamin A is phosphorylated by PAK1 on Serine 2152 (Vadlamudi, et al., 2002), we wanted to determine if JAK2 phosphorylation of PAK1, increasing PAK1 kinase activity (Rider, et al., 2007), would increase in Filamin A S\(^{2152}\) phosphorylation. 293T cells were transfected with Filamin A, with or without PAK1, wild type JAK2 or with kinase-inactive JAK2 (KE). Cell lysates were prepared, proteins were separated on an SDS-PAGE gel, transferred and the membrane was probed with anti phospho-Filamin A antibody (specific for phosphorylation on Serine 2152). Filamin A phosphorylation was increased when wild type JAK2 cDNA was co-transfected with PAK1 cDNA (Figure 6, lane 6 compared to 5). A similar effect was observed when Filamin A was immunoprecipitated (lane 2 compared to 1). There is background phosphorylation in the lanes with either PAK1 and Filamin A or JAK2 and Filamin A which could be due to PAK-independent phosphorylation of Filamin A (lanes 1, 3, 5, and 7). To confirm that this increase in Filamin A S\(^{2152}\) phosphorylation was dependent on JAK2 phosphorylation of PAK1 we used the kinase-inactive JAK2 and did not see the increase in Filamin A.
<table>
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<td>Vadlamudi, et al. 2002</td>
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<td>Increases LIMK-1 phosphorylation and then phosphorylation of cofilin</td>
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<td>Merlin</td>
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<tr>
<td>p41-Arc</td>
<td>Required for cell motility</td>
<td>Vadlamudi, et al. 2004</td>
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**Figure 5: Cytoskeletal targets of PAK1.** Each target listed is phosphorylated by PAK1 and the effect of that phosphorylation is listed.
Figure 6: JAK2-dependent phosphorylation of PAK1 increases Filamin A S2152 phosphorylation. 293T cells were transfected with Filamin A-myc, with or without PAK1-HA, and WT JAK2 or Kinase-Inactive JAK2 (KE). Filamin A-myc was immunoprecipitated from cell lysates with α-myc antibody. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with the listed antibodies. Lysate and IP lanes were run on the same gel, blots are cropped because they show different times of exposure of the ECL reaction to film.
phosphorylation (lanes 4 compared to 2 or 8 compared to 6). Actually, there is a decrease in Filamin A S\textsuperscript{2152} phosphorylation with kinase-inactive JAK2 compared to the lane with only PAK1 and Filamin A (lanes 4 and 8 compared to 1 and 5 respectively). This decrease is probably due to the decreased PAK1 expression in lane 8. The membrane was re-probed with anti-myc for total myc-tagged Filamin A to demonstrate that the total protein amount was similar throughout the lanes and was not the reason for the increase in phosphorylation signal. It was also reprobed with the listed antibodies to detect the levels of protein expression. It is important to note that there is an unspecific band in lane 3 of the anti-HA blot. It is unclear what this band is, because there is no band in the lysate lane which confirms that this band cannot be due to HA-tagged PAK1.

**Filamin A is Tyrosyl-phosphorylated in a JAK2-dependent Manner**

There are several kinases that phosphorylate Filamin A on S\textsuperscript{2152} or S\textsuperscript{2523}; however, there are no data on tyrosyl-phosphorylation of Filamin A. Thus, we decided to test if Filamin A is phosphorylated on tyrosines in addition to the known serines. We were especially interested in JAK2-dependent phosphorylation of Filamin A because JAK2 is an important signaling molecule because of the number of ligands that are able to activate it. We found that Filamin A was tyrosyl-phosphorylated when 293T cells were transfected with Filamin A and JAK2 (Figure 7, lane 3). Also, the tyrosyl-phosphorylation of Filamin A was increased when PAK1 was co-transfected with Filamin A and JAK2 (lane 2 compared to 3). Since we know that PAK1 binds to Filamin A, our data suggest that PAK1 may recruit Filamin A to JAK2 so that it can be tyrosyl-phosphorylated because more Filamin A is tyrosyl-phosphorylated in the presence of
Figure 7: JAK2-dependent tyrosyl-phosphorylation of Filamin A is increased by PAK1. 293T cells were transfected with Filamin A-myc, with WT PAK1-HA or PAK1 Y3F, and WT JAK2 or Kinase-Inactive JAK2 (KE). Proteins from cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with the listed antibodies.
PAK1. To confirm that the tyrosyl-phosphorylation of Filamin A was JAK2-dependent we transfected cells with kinase-inactive JAK2 and PAK1 (wild type or Y3F) along with Filamin A, or simply PAK1 and Filamin A, and saw no detectable tyrosyl-phosphorylation of Filamin A (lanes 4, 6, and 1 respectively). Also, as expected, we did not see any difference in tyrosyl-phosphorylation of Filamin A between lanes containing wild type PAK1 and the mutant PAK Y3F (lane 2 compared to 5) because Filamin A binds to PAK1 at the CRIB domain, which is at the N-terminus and does not contain any of these three tyrosines. Therefore, a mutation at these sites should not affect the amount of tyrosyl-phosphorylated Filamin A because presumably Filamin A can still bind to PAK1 and be recruited to JAK2. In fact we did not observe any difference in the amount of tyrosyl-phosphorylated Filamin A.

**SH2B1β Does Not Enhance PAK1/JAK2-dependent Phosphorylation of Filamin A**

The adaptor protein SH2B1β is an enhancer of JAK2 activity and in turn, JAK2-dependent PAK1 activity (Rui, et al., 1997; Rider, et al., 2007). In order to test whether SH2B1β would further increase the PAK1/JAK2-dependent phosphorylation of Filamin A S2152, we transfected 293T cells with Filamin A, JAK2, PAK1 and SH2B1β. However, when SH2B1β was transfected along with Filamin A, JAK2, and PAK1 the Filamin A S2152 phosphorylation was lower than when only Filamin A, JAK2 and PAK1 were transfected (Figure 8, lanes 6 compared to 2 and 14 compared to 10). Additionally, Filamin A S2152 phosphorylation was increased in the lane with Filamin A, JAK2, and SH2B1β compared to the lane with only Filamin A and JAK2 (lanes 7 compared to 3 and 15 compared to 11). This suggests that SH2B1β may be competing with PAK1 for
Figure 8: SH2B1β does not enhance PAK1-dependent phosphorylation of Filamin A S2152. 293T cells were transfected with Filamin A-myc, with or without PAK1-HA, WT JAK2 or Kinase-Inactive JAK2 (KE), and with or without SH2B1β-GFP. Filamin A-myc was immunoprecipitated from cell lysates with α-myc antibody. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with the listed antibodies.
binding to Filamin A and SH2B1β may be recruiting a serine kinase other than PAK1 to phosphorylate Filamin A $S^{2152}$. When the membrane was reprobed with anti-HA we again saw the unspecific band that was described previously for figure 6.

**Endogenous and Overexpressed Filamin A Associate with Endogenous and Overexpressed SH2B1β in 293T and 3T3 F442A Cells**

If SH2B1β is competing with PAK1 for binding to Filamin A we would expect that Filamin A and SH2B1β associate with each other. To test this, 293T cells were transfected with Filamin A and SH2B1β and Filamin A was immunoprecipitated and immunoprecipitates were tested for presence of SH2B1β by blotting with anti-SH2B1. These preliminary data demonstrate that Filamin A and SH2B1β associate with each other (Figure 9, lane 1).

Since we observed an association of Filamin A and SH2B1β in a transient transfection system, we wanted to confirm that these proteins associate at endogenous levels. Also, previous studies showed that Lnk (SH2B3), a member of the SH2B family, and Filamin A bind to each other (He, et al., 2000). In order to determine if Filamin A and SH2B1β associate with each other, cell lysates from 293T and 3T3 F442A cells were incubated with antibody to SH2B1β. After the immunoprecipitated proteins were run on a gel and transferred to PVDF membrane, the membrane was blotted for anti-Filamin A. We observed that endogenous Filamin A was co-immunoprecipitated with endogenous SH2B1β in both cell lines (Figure 10A, lane 1 and 10B, lane 1). This co-immunoprecipitation confirms that Filamin A and SH2B1β can associate with each other.
Figure 9: SH2B1β associates with Filamin A. 293T cells were transfected with Filamin A-myc with or without SH2B1β-GFP. Filamin A-myc was immunoprecipitated from cell lysates with α-myc antibody. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with α-SH2B1 and reprobed with α-myc.
Figure 10: Endogenous SH2B1β associates with endogenous Filamin A in 293T and 3T3 F442A cells. (A) Endogenous SH2B1 was immunoprecipitated from 293T cell lysates using α-SH2B1 antibody. The proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with α-Filamin A and reprobed with α-SH2B1. (B) Endogenous SH2B1 was immunoprecipitated from 3T3 F442acell lysates as in (A).
**Filamin A Binds Directly to SH2B1β**

Since we demonstrated the association of Filamin A and SH2B1β, we wanted to determine if Filamin A and SH2B1β were directly binding to each other. In order to determine direct binding, we purified both Filamin A and SH2B1β. We used *in vitro* translation to generate purified Filamin A with $^{35}$S-Methionine incorporated for protein detection (Figure 11A). For purified SH2B1β we used affinity purified GST- SH2B1β attached to glutathione agarose beads. When $[^{35}\text{S}]$Filamin A was incubated with GST- SH2B1β attached to beads, the GST- SH2B1β pulled down $[^{35}\text{S}]$Filamin A greater than the background pull down with GST alone attached to beads (Figure 11B lane 2 compared to 1). This shows that Filamin A and SH2B1β are binding directly to each other. Now that we know that Filamin A and SH2B1β are directly binding, we wanted to determine the importance of this binding.

**JAK2-dependent Tyrosyl-phosphorylation of Filamin A is Facilitated by SH2B1β**

Since SH2B1β is an important adaptor protein that increases JAK2 activation, we were interested in the effect of SH2B1β on JAK2-depedent tyrosyl-phosphorylation of Filamin A. If SH2B1β can bind to JAK2 and Filamin A, we expect that SH2B1β may be able to recruit Filamin A to JAK2 where it will be tyrosyl-phosphorylated. When 293T cells were transfected with Filamin A, wild type JAK2 and SH2B1β we demonstrated that Filamin A was tyrosyl-phosphorylated (Figure 12, lane 4 and 10). There was no detectable phosphorylation when cells were transfected with only Filamin A and SH2B1β or Filamin A and kinase-inactive JAK2 with or without SH2B1β (lanes 2, 5 and 6). We also observed that SH2B1β was co-immunoprecipitated with Filamin A at similar levels.
**Figure 11: SH2B1β binds to Filamin A in vitro.** (A) Filamin A-myc and Luciferase (control) were translated *in vitro* with the addition of $^{35}$S-Methionine. Lane 1: Luciferase, Lane 2: Filamin A-myc. (B) *In vitro* translated Filamin A-myc was incubated with GST or GST-SH2B1β attached to glutathione beads. The proteins were eluted from the beads, separated by SDS-PAGE, transferred to PVDF membrane and exposed to film to detect the $^{35}$S signal. The membrane was also immunoblotted with α-myc and α-GST. Lane 1: GST, Lane 2: GST-SH2B1β.
Figure 12: SH2B1β enhances JAK2-dependent tyrosyl-phosphorylation of Filamin A. 293T cells were transfected with Filamin A-myc, with WT JAK2 or Kinase-Inactive JAK2 (KE), and with or without SH2B1β-GFP. Filamin A-myc was immunoprecipitated from cell lysates with α-myc antibody. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted with the listed antibodies.
whether JAK2 was present or not, or even in its kinase-inactive form. Similar amounts of SH2B1β co-immunoprecipitated suggests that Filamin A binding to SH2B1β is independent of the phosphorylation state of SH2B1β. This data suggests that SH2B1β can recruit Filamin A to JAK2 for tyrosyl-phosphorylation even if SH2B1β has not yet been activated by JAK2.

Summary

We have demonstrated that JAK2 phosphorylation of PAK1 increases cell motility of Human Mammary Epithelial cells. To begin to elucidate a mechanism of tyrosyl-phosphorylated PAK1-dependent cell motility, we demonstrated that when PAK1 is tyrosyl-phosphorylated by JAK2 it increases phosphorylation of Filamin A S2152. This phosphorylation is required for PAK1 mediated actin-cytoskeletal reorganization (Vadlamudi, et al., 2002). We have for the first time demonstrated that Filamin A is tyrosyl-phosphorylated in a JAK-dependent manner and that JAK2-dependent phosphorylation of Filamin A is enhanced by PAK1. We also demonstrated that the adaptor protein SH2B1β, a target of JAK2, directly binds to Filamin A in vitro and both overexpressed and endogenous SH2B1β binds to over-expressed and endogenous Filamin A in vivo. Although SH2B1β does not enhance PAK1-dependent phosphorylation of Filamin A on S2152, SH2B1β facilitates JAK2-dependent tyrosyl-phosphorylation of Filamin A.
Discussion

PAK1 has been implicated in increasing cell motility, but the connection between the upstream regulators and downstream targets of the PAK1 involvement in cell motility has not been elucidated. One possible upstream regulator of PAK1 that may be involved in cell motility is the tyrosine kinase JAK2 because JAK2-dependent phosphorylation of PAK1 increases PAK1 kinase activity and also protects cells from apoptosis (Rider, et al. 2007). We investigated here whether PAK1 activation by JAK2 increased cell motility. Maximal cell motility in HME cells occurred only when wild type PAK1 and wild type JAK2 were contransfected or when the constitutively active mutant of PAK1 was transfected. The increase in phagokinetic index is significant, but it is small because we used epithelial cells, which are not normally motile. We expect that the difference would have been much greater if we had used more motile cells, for example fibroblasts. This increase in cell motility confirms that activated PAK1 increases cell motility and that JAK2 is one way that PAK1 can be activated to alter downstream phenotypes.

We then sought to understand the mechanism that causes the increase in cell motility due to increased JAK2-dependent PAK1 activity. After examining several known downstream cytoskeletal targets of PAK1, we observed an increase in Filamin A phosphorylation on S\textsuperscript{2152} that was dependent on JAK2 phosphorylation of PAK1. We concluded that the increase in S\textsuperscript{2152} phosphorylation is dependent on JAK2 because this increase is not present if we use a kinase-inactive form of JAK2. We also concluded that
the increase in $S^{2152}$ phosphorylation is dependent on PAK1 and that JAK2 is not activating another serine kinase because there is no increase in $S^{2152}$ phosphorylation when JAK2 is transfected without PAK1. There is a background level of Filamin A phosphorylation in the lane with PAK1 alone; this could be due to PAK1 activated by another mechanism (e.g., small GTPases), endogenous JAK2, or another kinase that can phosphorylate Filamin A on $S^{2152}$. We attribute the increase in Filamin A phosphorylation to the increase in PAK1 kinase activity when PAK1 is phosphorylated by JAK2. Still, it is unclear how Filamin A phosphorylation is involved in actin reorganization. It is known however, that Filamin A phosphorylation on $S^{2152}$ is required for PAK1-dependent ruffle formation as well as for Ribosomal S6 Kinase-dependent motility (Vadlamudi, et al., 2002; Woo, et al., 2004). Since phosphorylation on $S^{2152}$ by Ribosomal S6 Kinase is important for cell motility, we believe that phosphorylation at this site by PAK1 may be another means of regulating cell motility.

Tyrosine kinase JAK2 is activated by more than two-thirds of the ligands that belong to receptors of the cytokine hematopoietin superfamily. Because JAK2 is involved in signaling from many ligands and is involved in many cellular outcomes, there are many targets that are phosphorylated by JAK2. We were interested in whether Filamin A was a downstream target of JAK2. Interestingly, we found that Filamin A is tyrosyl-phosphorylated in a JAK2 dependent manner. Also, tyrosyl-phosphorylation is increased in the presence of PAK1 suggesting that PAK1 recruits Filamin A to activated JAK2 which is associated with a receptor. It is unclear at this time the functional relevance of this tyrosyl-phosphorylation of Filamin A, however, since Filamin A is an actin-
regulating protein, we anticipate that tyrosyl-phosphorylation of Filamin A may be an additional level of regulation of the actin cytoskeleton.

We decided to further characterize the increase in Filamin A S^{2152} phosphorylation by PAK1. Since JAK2, and thus PAK1, activity can be increased by the adaptor protein SH2B1β, we expected to see a further increase in Filamin A S^{2152} phosphorylation when SH2B1β was transfected along with PAK1 and JAK2. Surprisingly, we saw a decrease in JAK2/PAK1-dependent Filamin A phosphorylation on S^{2152} in the presence of SH2B1β. Also there is an increase in Filamin A S^{2152} phosphorylation where JAK2 and SH2B1β are transfected without PAK1. The decrease in S^{2152} phosphorylation in the presence of SH2B1β suggests that SH2B1β may be competing with PAK1 for binding to Filamin A. When SH2B1β is present, less PAK1 can bind to and phosphorylate Filamin A on S^{2152}. Also, the increase in PAK1-independent phosphorylation of Filamin A S^{2152} suggests that SH2B1β may be recruiting another serine kinase to Filamin A to facilitate phosphorylation on S^{2152}.

If SH2B1β has an effect on Filamin A phosphorylation, we would expect that SH2B1β can bind to Filamin A because SH2B1β is an adaptor protein and has no kinase activity. SH2B3 (Lnk), an SH2B family member that has much homology to SH2B1β, can bind to Filamin A (He, et al., 2000). The homology between SH2B1β and SH2B3 supports the hypothesis that SH2B1β could bind to Filamin A. We were able to confirm that both endogenous and overexpressed SH2B1β associates with endogenous and overexpressed Filamin A in 293T and 3T3 F442A cells. Also, purified SH2B1β and Filamin A bind to each other in vitro confirming that these proteins are binding directly to each other.
We next wanted to understand the function of SH2B1β binding to Filamin A. Since SH2B1β binds to JAK2 and increases its activity, we hypothesized that SH2B1β may increase JAK2-dependent tyrosyl-phosphorylation of Filamin A. In fact, we observed that SH2B1β does increase tyrosyl-phosphorylation of Filamin A suggesting that SH2B1β recruits Filamin A to JAK2 where it is then tyrosyl-phosphorylated. The tyrosyl-phosphorylation of Filamin A may be a mechanism to increase the effects that have already been described for Filamin A phosphorylation on S\textsuperscript{2152} or my serve an entirely different function that is currently unknown.

We have demonstrated a possible connection between upstream events that phosphorylate and activate PAK1 with downstream events that increase cell motility. We suggest a model where Filamin A is regulated by either the JAK2/PAK1 complex or the JAK2/SH2B1β complex (Figure 13). In this model, PAK1 phosphorylates Filamin A on S\textsuperscript{2152} and recruits Filamin A to JAK2 where Filamin A is then tyrosyl-phosphorylated (Figure 13A). Also, Filamin A can bind to SH2B1β which also recruits Filamin A to JAK2 where Filamin A is tyrosyl-phosphorylated (Figure 13B). We suggest these two separate models because our data suggest that all four proteins do not form a single complex because of PAK1 and SH2B1β competing for binding of Filamin A. S\textsuperscript{2152} phosphorylation and tyrosine phosphorylation are two possible ways that Filamin A can be regulated. We anticipate that both serine and tyrosine phosphorylation of Filamin A may be involved in actin reorganization. It is not yet understood how phosphorylation of Filamin A increases actin reorganization. One possible explanation is that phosphorylation of Filamin A alters the amount of Filamin A that can bind to actin to allow for the breakdown and reorganization of actin gels. Another possibility is that
Figure 13: Proposed model of Filamin A phosphorylation. (A) PAK1 recruits Filamin A to JAK2 where Filamin A is tyrosyl-phosphorylated in a JAK2-dependent manner and Filamin A is phosphorylated on S2152 by PAK1. (B) SH2B1β recruits Filamin A to JAK2 where both Filamin A and SH2B1β are tyrosyl-phosphorylated in a JAK2-dependent manner.
phosphorylated Filamin A can associate with other actin regulating proteins to facilitate actin reorganization.

We have many future plans that will expand our knowledge of the Filamin A interaction with PAK1, JAK2, and SH2B1β and hopefully allow us to make a connection between Filamin A phosphorylation and actin reorganization and cell motility. We plan to examine whether PAK1-dependent increased Filamin A phosphorylation increases cell motility. We also want to determine if Filamin A and PAK1 localization/cocolocalization changes when PAK1 is activated by JAK2. We would like to map the sites of Filamin A tyrosyl-phosphorylation as well as the sites in Filamin A and SH2B1β that are necessary for their association. It will also be helpful to understand where Filamin A and SH2B1β colocalize to see if the colocalization occurs in areas of high actin reorganization.

Understanding this colocalization may help in further understanding the relevance of Filamin A and SH2B1β binding to each other in terms of actin regulation.


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