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Publication Date
2010

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The Dynamic Interplay between Adhesion Signaling and the ERK MAP Kinase Pathway

in

Molecular Pathology

by

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2010
The Dissertation of Jacob Robert Haling is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego
2010
DEDICATION

For my mom, dad, and my wife Breanna, who instilled within me the pursuit of knowledge and the love of life.
Education: that which reveals to the wise, and conceals from the stupid, the vast limits of their knowledge.

Mark Twain
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ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge Mark Ginsberg for his mentoring and support as the chair of my committee. Mark has been instrumental in my development as a scientist, starting from how to write a protocol and culminating with this dissertation, with successful pre-doctoral fellowships and publications along the way. Although I frequently dreaded our weekly data meetings, Mark has taught me to think critically about my data and how to design the next experiment to get a useful answer. His guidance has proved to be invaluable, and I am grateful for him taking me on to be one of his graduate students.

I would like to thank my committee members, Sanford Shattil, Daniel Donoghue, Richard Klemke, Robert Liddington, and Geert Schmid-Schonbein for taking the time to meet with me and help shape this project. Sandy, in particular has provided valuable feedback through our Thursday group meetings.

I would like to acknowledge Larry Goldfinger for his guidance for my first few years in the lab. Larry took me in under his wing when I first joined the lab and set aside his valuable time to teach me several lab techniques and answer my naïve questions about projects, papers, or life in general. I would not have survived in the lab if it wasn’t for Larry.

I would also like to acknowledge Brian Petrich, Feng Ye, and Joe Cantor for taking the time to read, edit, and critique multiple drafts of my dissertation. I would like to also thank them for our many scientific discussions over the years that have led to successful experiments and have saved other experiments from
certain disaster. Their support has helped me in an immeasurable way. More importantly, I have enjoyed our endless debates about sports, and although I am usually right, they have proven to be formidable adversaries.

I would like to acknowledge my mom and dad, for their support and encouragement. My parents provided me with a strong foundation at an early age to ask questions, to challenge accepted ideas, and to believe in myself. I would not be where I am today if they did not stress the importance of education. My brothers, Jonathan and Andrew, have also been a source of inspiration, and watching them succeed in their respective career paths has made me a proud older brother.

Finally, I would like to acknowledge the most important person in my life, my wife Breanna. I met her at the same time I started graduate school and she has shared in every one of my triumphs and failures. At times when I wanted to quit graduate school, she reminded me of my passion for science and kept me focused on the larger picture. She was also my biggest fan and was there to celebrate my minor accomplishments along the way. Breanna has accommodated my long hours in the laboratory and never-ending work load, and for this I am forever grateful.

Chapter 2, in full, is a reprint of the material as it appears in Molecular Biology of the Cell, 21 (4), 664-73 (2010). Haling, Jacob R; Wang, Fen; Ginsberg, Mark H. Jacob Haling was the primary investigator and author of this paper.
Chapter 3, in full, is a reprint of the material as it appears in The Journal of Biological Chemistry, 284(52), 36700-10 (2009). Anthis, N; Haling, JR; Oxley, CL; Memo, M; Wegener, KL; Lim, CJ; Ginsberg, MH; Campbell, ID. Jacob Haling was the secondary investigator and author of this paper.
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ABSTRACTS


Mitogen-activated protein (MAP) kinases and integrins are involved in a variety of processes including but not limited to, cell proliferation, differentiation, survival, and cell migration. Thus it is not surprising that aberrant signaling by either of these elements has developmental and pathological consequences. Whereas, MAP kinases and integrins coordinate these important cellular processes there is a lack of insight into how these two signaling components
intersect. In this dissertation I employ a variety of biochemical, molecular biology, and cell biological techniques to decipher three distinct interfaces between integrins and MAP kinases. First, I will demonstrate how PEA-15 potentiates MAP kinase signaling by limiting ERK1/2 association with the plasma membrane. Next I will detail how tyrosine phosphorylation of beta integrin cytoplasmic tails regulates the binding of intracellular binding proteins. Lastly, I will explain how ERK1/2 suppresses integrin activation through the adaptor protein FRS2α. In addition, I will provide a biological context for the experimental results discussed in this dissertation.
Mitogen-activated protein (MAP) kinases and integrins are both involved in a variety of processes, including but not limited to, cell proliferation, differentiation, survival, and cell migration. Thus it is not surprising that aberrant signaling by either of these pathways has developmental and pathological consequences. The importance of regulating MAP kinase and integrin signaling is especially evident in cancer. Over one-third of all cancers contain an activating mutation in Ras, a key member of the ERK1/2 MAP kinase pathway, with pancreas (90%), colon (50%), thyroid (50%), and lung (30%) having the highest prevalence (Malumbres and Barbacid, 2003). In addition, mutations in B-Raf are present in 66% of malignant melanomas (Rushworth et al., 2006) and mutations in epidermal growth factor receptor (EGFR) are found in 80% of non-small cell lung cancers (Dy and Adjei, 2009) indicating that proper function of the ERK1/2 MAP kinase pathway is critical for normal physiological function. Furthermore, tumor metastasis promotes the spread of cancer to local and distant sites.

Integrins can also contribute to tumor formation and metastasis. Increased levels of integrin expression are associated with the metastatic nature of a tumor (Jin and Varner, 2004). For example α6β4 enhances tumor cell invasiveness in breast carcinomas (Ramos et al., 2002), αvβ3 is expressed on invasive melanoma whereas it is not expressed on normal melanocytes (Gehlsen
et al., 1992), and α6 is upregulated in numerous cancers (Mercurio et al., 2001). Antagonists to αvβ3 and αvβ5 (e.g. etaracizumab, CNTO 95, Cilengitide) have had promising results in clinical trials in cancer patients (Desgrosellier and Cheresh, 2010). Additionally, a function blocking antibody against α5β1 (Volociximab) inhibits angiogenesis and impedes tumor growth of human breast cancer (Kuwada et al., 2007). Thus, integrins are accessible drug targets that function in tumor progression and metastasis.

MAP kinase and integrin signaling are not only important for the same cellular behaviors and converse pathologies, but they are linked biochemically through partially-shared signal transduction pathways. For example, alterations in integrin affinity for extracellular ligand are important for regulating cell migration and adhesion. An increase in integrin-mediated affinity inhibits cell migration (Huttenlocher et al., 1996), whereas an increase in ERK1/2 MAP kinase signaling enhances cell migration (Klemke et al., 1997). Furthermore, the rear detachment of a cell is the rate limiting step of cell migration, and this process is dependent on the release of integrin adhesion (Palecek et al., 1998) and ERK1/2 mediated cell contraction (Stupack et al., 2000). Interestingly, HRas, Raf-1, MEK, and membrane localized ERK of the ERK1/2 MAP kinase pathway inhibit integrin activation (Hughes et al., 1997; Ramos et al., 2000; Chou et al., 2003) and thus provide a relationship between MAP kinase signaling and integrin activation in the context of cell motility. Here I will discuss the current progress and open questions about mechanisms that regulate integrin and MAP
kinase signaling. In addition, I will introduce the molecular players and provide a biological context for the experimental results discussed in this dissertation.

**MAP Kinase Signaling**

In mammals, there are more than a dozen MAP kinase genes encoding extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 (α, β, γ, and δ), c-Jun N-terminal kinase (JNK1-3), and the more recently discovered big mitogen-activated protein kinase-1 (BMK1 or ERK5). Each of these 4 families of MAP kinases has a similar activation hierarchy consisting of three components: a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MAP2K), and a MAP kinase (MAPK). MAP3Ks phosphorylate and activate MAP2Ks, which then phosphorylate and activate MAPKs. This allows amplification of extracellular signals and a tighter control of the signaling pathway. Furthermore, in a multi-tiered pathway the subcellular localization of each of the kinases and the kinetics of their activation can control the outcome of their signals. The diversity of MAP kinase signaling allows a specific cellular response from a particular stimulus. Here I will briefly discuss p38, JNK, and ERK5 before describing the ERK1/2 MAP kinase cascade in more detail.

The p38 and JNK MAP kinases are commonly referred to as the pro-inflammatory and cellular stress MAP kinases due to the events that activate them. p38 was originally discovered as a protein that is phosphorylated in cells treated with lipopolysaccharide (Han et al., 1994) and bound to pyridinyl imidazole compounds (Lee et al., 1994). These drugs inhibit IL-1 and TNF
production from stimulated human monocytes, and were later found to do so by blocking the p38 kinase. The p38 family members are activated by extracellular cues such as UV radiation, osmotic shock, hypoxia, and pro-inflammatory cytokines. Activated p38 MAP kinase potentiates inflammatory cytokine production by direct phosphorylation of transcription factors (e.g. ATF2, CREB) and by stabilizing translation of mRNA’s encoding pro-inflammatory cytokines (Schieven, 2005). The p38 MAP kinase is up-regulated in several diseases initiated or accompanied by chronic inflammation, such as rheumatoid arthritis and Alzheimer’s disease (Munoz and Ammit, 2009). Recently, there has been an effort to design specific inhibitors of p38 MAP kinases for better treatment of these diseases (Dominguez et al., 2005). Blocking p38 MAP kinase with SB-203580 blocks smooth muscle cell migration by preventing phosphorylation of HSP27 (Hedges et al., 1999), which is a protein that promotes actin remodeling by enhancing actin phosphorylation. This function is necessary in smooth muscle cells for the proliferative response to injury (Gerthoffer et al., 2001).

The JNK MAP kinases are activated by similar extracellular stimuli as p38 MAP kinases, yet can generate a different response within the cell. One of the substrates of JNK kinases is the transcription factor AP1, which is composed of Jun and Fos family members (Eferl and Wagner, 2003). These are proteins that regulate the cell cycle, apoptosis, and cell-survival (Altucci and Gronemeyer, 2001). Therefore, it is no surprise that mutations in the JNK pathway can result in cancers (Wagner and Nebreda, 2009). Recent work has demonstrated that active JNK has functions in the cytosol as well. For example JNK
phosphorylates the focal adhesion adaptor protein paxillin, and this may promote paxillin degradation, focal adhesion disassembly, and ultimately increased cell migration (Huang et al., 2004). Thus, both JNK and p38 have non-nuclear targets that influence how a cell interacts with its extracellular environment.

Recently, the ERK5 MAP kinase pathway was discovered and added as the fourth member of the MAP kinase family (Zhou et al., 1995). It is sometimes referred to as the big MAP kinase due to its large size of 816 amino acids (Zhou et al., 1995; Yan et al., 2001). Genetic deletion of ERK5 is embryonically lethal due to cardiovascular defects. Conditional knockouts reveal that deletion of ERK5 in cardiomyocytes does not effect development, whereas deletion of ERK5 in endothelial cells gives the same phenotype as a global knockout (Hayashi et al., 2004). This established that ERK5 plays an important role in cardiac function through endothelial cells. In addition to being activated by a limited number of growth factors (Kato et al., 1997; Kesavan et al., 2004), ERK5 is also activated by conditions such as fluid shear stress, hypoxia, and ischemia (Yan et al., 1999; Sohn et al., 2002; Takeishi et al., 1999). Substrates of ERK5 are still being identified, but ERK5 does share some common nuclear substrates with the classical MAP kinases (Nishimoto et al., 2006) that aid in inhibiting endothelial apoptosis (Pi et al., 2004). Recently, ERK5 has been reported to have a functional relationship with integrin αVβ3 and focal adhesion kinase (FAK) (Sawhney et al., 2008). FAK’s role in MAP kinase signaling will be discussed later in greater detail, but in short, integrins that bind to vitronectin activate FAK which in turn activates ERK5 signaling. Active ERK5 has been suggested to
promote cell motility, but this mechanism is poorly understood. Much work is needed to identify unique substrates for ERK5 in response to physiological and pathological conditions in order to fully understand the specific role of this MAP kinase.

ERK1 (p44) and ERK2 (p42) are 84% identical and have overlapping functions within the cell (Lloyd, 2006), hence they will be referred to as ERK1/2 for the remainder of this report. The amount of research encompassing this particular pathway may have one wondering if ERK1/2 has its proverbial hand in every function of the cell ranging from cell cycle progression and cell death to GAP junction formation. Thus it may not be surprising that ERK1/2 can phosphorylate over 100 possible substrates, or that ERK1/2 has vital roles in heart development, memory formation, immune function, and many other physiological processes. Dysfunction of ERK1/2 signaling has dire pathological consequences including cancer, cardiovascular disease, and diabetes. Despite the association of ERK1/2 with a variety of biological functions or diseases, the mechanisms that govern many of these events are incomplete or poorly understood. I will make an attempt to summarize what is known about the ERK1/2 MAP kinase pathway and introduce some open questions that remain along the way.

ERK1/2 is activated by a variety of signaling events including growth factor receptors, integrins, and G-protein coupled receptors (GPCR). The canonical ERK1/2 MAP kinase cascade, which will be discussed in depth here, refers to the binding of receptor tyrosine kinases (RTKs) to growth factors which activate a
signaling pathway leading to ERK1/2 activation. RTKs are transmembrane proteins that contain an N-terminal extracellular ligand binding domain and a C-terminal intracellular kinase domain (Schlessinger, 2000; Pawson, 2002). Upon the binding of ligand/growth factor, dimerization of the RTK occurs and induces autophosphorylation of the tyrosine residues on the intracellular tyrosine kinase domain. Tyrosine phosphorylation of the C-terminal kinase domain serves as a docking site for proteins containing either a phosphotyrosine binding (PTB) domain or a Src-homology (SH2) domain. A variety of different proteins can bind to these docking sites and fall into three categories: (1) enzymes (e.g. PI3K, Src, and PLCγ), (2) adaptors (e.g. Grb2 and Shc), and (3) docking proteins (e.g. IRS, FRS2, and Dok proteins). Docking proteins contain either a pleckstrin homology (PH) domain or a myristoylation site for localization to the plasma membrane, and are recruited to RTKs through an intrinsic PTB domain or interaction with the Grb2 adaptor.

Activation of the small GTPase Ras is the key link between RTKs and ERK1/2. Ras is activated at the plasma membrane by the SOS-Grb2 adaptor. Grb2 is recruited from the cytosol to the plasma membrane upon growth factor stimulation, where its SH2 domain can bind to either a docking protein (e.g. FRS2) or directly to the tyrosine phosphorylated RTK. Grb2 is constitutively bound to the Ras guanine nucleotide exchange factor SOS, which then promotes GTP loading and activation of Ras at the plasma membrane. Next, Raf translocates to the plasma membrane and binds to GTP-loaded Ras resulting in a catalytically active conformation of this MAP3K. MEK (MAP2K) is a dual
specificity kinase that binds to inactive ERK1/2 and retains it in the cytoplasm. Upon Raf activation of MEK, MEK phosphorylates ERK1/2 at threonine and tyrosine residues of the conserved TEY motif within the phosphorylation loop. Active ERK1/2 is released from MEK, where it can then translocate to the nucleus for transcriptional activities or act upon a separate set of substrates in the cytoplasm or at the plasma membrane.

Attenuation and regulation of the ERK1/2 MAP kinase pathway is achieved by phosphatases, scaffolding proteins, localization, and feedback loops. Dephosphorylation of either the threonine or tyrosine residue of the ERK1/2 activation loop TEY leads to complete enzymatic inactivation (Posada et al., 1992). In addition to tyrosine phosphatases and serine/threonine phosphatases there are phosphatases capable of dephosphorylating both of these sites and are called dual-specificity phosphatases (DSP) or MAP kinase phosphatases (MKPs). MKPs specific for ERK1/2 exist in the cytoplasm and nucleus to provide a range of negative feedback (Keyse, 2008). The down-regulation or inactivity of MKPs specific for ERK1/2 can result in pancreatic carcinoma (Furukawa et al., 2003). Considering that Raf and MEK are also activated by phosphorylation, phosphatases are capable of regulating these kinases as well. Recently, a computational model outlining the functional relevance of phosphorylation and dephosphorylation suggested that Raf dephosphorylation, MEK phosphorylation, and MEK dephosphorylation are the most important reactions controlling the propagation of the ERK1/2 MAP kinase pathway (Hornberg et al., 2005). The serine/threonine specific phosphatase
PP2A regulates ERK1/2 activation by interacting directly with ERK1/2 (Sontag et al., 1993) or with upstream activator proteins. PP2A can bind to the adaptor protein Shc, thereby preventing its tyrosine phosphorylation and the activation of Ras (Ugi et al., 2002). A necessary component required for ERK1/2 activation. Interestingly, PP2A positively mediates signaling from Raf. PP2A functions to dephosphorylate key sites on the scaffold protein kinase suppressor of Ras (KSR) and Raf, facilitating their recruitment to the membrane for activation (Ory et al., 2003). Thus PP2A can have both a positive and negative role in ERK1/2 signaling.

The localization of ERK1/2 is important component in controlling its activity. Retention of active ERK1/2 in the cytoplasm blocks growth factor-induced proliferation (Brunet et al., 1999) and cell transformation (Robinson et al., 1998). This is due to inhibiting ERK1/2 transcriptional activities. The shuttling of ERK1/2 between the nucleus and cytoplasm is still largely not understood. What is known is that MEK contains a nuclear export sequence (NES) and remains bound to ERK1/2 under quiescent conditions. When MEK and ERK1/2 become phosphorylated via activation of the MAP kinase cascade, ERK1/2 is released from MEK and can then translocate into the nucleus. It has been suggested that ERK1/2 forms a homodimer when it is released from MEK, and this exposes a nuclear import sequence (Cobb and Goldsmith, 2000). In addition, MEK can facilitate the export of inactive ERK1/2 from the nucleus (Adachi et al., 2000).
Scaffold proteins also influence the localization of ERK1/2. While KSR was previously mentioned to be a key scaffold protein for plasma membrane activation of ERK1/2, ERK1/2 can be activated at other cellular compartments as well. MEK partner-1 (MP1) localizes specifically to endosomes, where it promotes and activates ERK1/2 in response to EGF (Teis et al., 2002). Active ERK1/2 at endosomes may participate in signaling through internalized cell surface receptors (Pouyssegur et al., 2002). Similar expression to FGF (Sef) is another spatial regulator of ERK1/2 MAP kinase signaling. Sef is a transmembrane protein that localizes and assembles active MEK and ERK1/2 to the Golgi apparatus (Torii et al., 2004). As a result of the formation of this complex, active ERK1/2 cannot enter the nucleus and can only phosphorylate cytoplasmic targets. Thus, KSR, MP1, and Sef are all examples of upstream scaffold proteins that permit ERK1/2 activation at precise cellular locations.

A well characterized downstream scaffold of ERK1/2 is phosphoprotein enriched in astrocytes of 15 kDa (PEA-15). PEA-15 was originally discovered in astrocytes (Araujo et al., 1993) and subsequently found to be widely expressed in a variety of tissues and is conserved among mammals (Danziger et al., 1995). PEA-15 comprises of an N-terminal death effector domain (DED) and a largely unstructured C-terminal tail (Hill et al., 2002). PEA-15 binds directly to ERK1/2 and limits ERK1/2 entry into the nucleus by blocking nuclear import and promoting nuclear export (Formstecher et al., 2001; Whitehurst et al., 2004). NMR "footprinting" and site-directed mutagenesis show that residues in the DED and in the tail of PEA-15 are involved in ERK1/2 binding (Formstecher et al.,
2001; Hill et al., 2002). Furthermore, protein kinase C and Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII)/AKT phosphorylate PEA-15 at Ser104 and Ser116, respectively, thus inhibiting ERK1/2 binding (Krueger et al., 2005). Whereas PEA-15 inhibits ERK1/2 phosphorylation of the nuclear transcription factor Elk-1, it does not inhibit the phosphorylation of ERK1/2 cytosolic targets such as stathmin or RSK2 (Formstecher et al., 2001; Vaidyanathan and Ramos, 2003; Krueger et al., 2005). Therefore, PEA-15 functions to redirect ERK1/2 signaling rather than to inhibit ERK1/2 intrinsic kinase activity. Paradoxically, the expression of PEA-15 enhances activation of Ras and thus MAP3K (Raf-1) and MEK that lead to ERK1/2 phosphorylation and activation (Ramos et al., 2000).

The mechanism by which PEA-15 potentiates the ERK1/2 MAP kinase pathway is not known. However, PEA-15 expression has been implicated in numerous pathologies, including glioma, breast cancer, ovarian cancer, astrogliosis, and diabetes (Bera et al., 1994; Hwang et al., 1997; Condorelli et al., 1998; Tsukamoto et al., 2000; Embury et al., 2001; Hao et al., 2001; Underhill et al., 2001; Sharif et al., 2004; Glading et al., 2007; Bartholomeusz et al., 2008).

Signal duration of ERK1/2 activation is of primary importance to determine a cellular response (Murphy and Blenis, 2006). For example, prolonged activation of ERK1/2 in PC12 cells is associated with differentiation, while transient activation of ERK1/2 is associated with proliferation in these cells (Marshall, 1995). Therefore, positive or negative feedback mechanisms can influence a cell's fate. ERK1/2 can phosphorylate MEK on two threonine residues, which prevents further MEK activity, and thereby attenuates ERK1/2
activation (Elben et al., 2004; Sundberg-Smith et al., 2005). In addition ERK1/2 can directly phosphorylate Raf on multiple sites, which has controversial results. One report observed that ERK1/2 phosphorylation of Raf further increases its activity up to 4 fold higher (Balan et al., 2006), whereas another report observed that ERK1/2 phosphorylation of Raf prevents its interaction with GTP loaded Ras (Dougherty et al., 2005) providing a negative feedback mechanism. What determines how ERK1/2 potentiates or inhibits Raf activity remains unresolved.

The plasma membrane linked docking protein FRS2α permits sustained activation of ERK1/2 and is involved in an ERK1/2-mediated negative feedback loop. Upon growth factor signaling FRS2α undergoes tyrosine phosphorylation, generating six functional pY-binding sites. These pY-binding sites function to form a complex with the tyrosine phosphatase Shp2 and the adapter protein Grb2, which associates with the Ras activator SOS. The FRS2α-Grb2-SOS ternary complex then activates the ERK1/2 MAP kinase cascade in a sustained manner (Wang et al., 1996; Kouhara et al., 1997; Hadari et al., 1998; Xu et al., 1998; Ong et al., 2000). Active ERK1/2 then phosphorylates FRS2α on eight threonine residues, leading to decreased pY of FRS2α and attenuation of the ERK1/2 MAP kinase pathway (Lax et al., 2002; Wu et al., 2003). Thus FRS2α and ERK1/2 form a negative feedback loop to limit MAP kinase activation in response to growth factor stimulation.

FRS2α, unlike MEK, Raf, or ERK1/2, is permanently bound to the plasma membrane and provides a unique mode of negative feedback. If ERK1/2 were prevented access to this adaptor protein, MAP kinase activation would be
prolonged. Considering that PEA-15 prevents ERK1/2 translocation into the nucleus, and yet increases MAP kinase signaling, we hypothesized that PEA-15 prevents ERK1/2 localization to the plasma membrane as well. This would prevent threonine phosphorylation of FRS2α and explain PEA-15’s ability to potentiate ERK1/2 MAP kinase activation. This hypothesis is tested in chapter 2.

**ERK1/2 Involvement in Focal Adhesion Dynamics**

Focal adhesions are sites of integrin clustering that link the actin cytoskeleton to the extracellular matrix (ECM) (Burridge *et al*., 1988). These sites are the control machinery of cell migration (Lock *et al*., 2008), and require equilibrium between actin polymerization and the continuous formation and disassembly of focal adhesions for normal cell motility (Gupton *et al*., 2006). The ERK1/2 MAP kinase cascade is involved in this function as well. ERK1/2 pathway inhibitors PD98059 and U0126 inhibit the migration of cells in response to growth factors or cell matrix proteins such as fibronectin, vitronectin, and collagen (Anand-Apte *et al*., 1997; Klemke *et al*., 1997; Xie *et al*., 1998; Shono *et al*., 2001). In addition, the over-expression of a dominant negative ERK mutant or knockdown of endogenous ERK1/2 also inhibits cell migration (Klemke *et al*., 1997; Lai *et al*., 2001). Several ERK1/2 substrates that are involved in cell migration have been identified. These substrates include paxillin and myosin light chain kinase (MLCK). Paxillin serves as an ERK1/2 scaffold protein that binds to integrins and associates with focal adhesion complexes (Ishibe *et al*., 2004; Kummer and Ginsberg, 2006). ERK1/2 mediated phosphorylation of
paxillin has been hypothesized to regulate FAK association with paxillin. Confocal microscopy reveals that growth factor stimulation results in distinct colocalization of FAK with ERK-phosphorylated paxillin in focal adhesions (Ishibe et al., 2004). The loss of ERK-mediated phosphorylation on paxillin dramatically slows cell migration in response to growth factor (Ishibe et al., 2004). The function of the ERK1/2-FAK-paxillin complex coordinates multiple interactions and regulates dynamic processes such as adhesion turnover and migration (Ishibe et al., 2004; Webb et al., 2004). One such interaction includes ERK1/2 phosphorylation of MLCK which in turn phosphorylates myosin light chain (MLC) and activates a cells’ motility machinery necessary for the polymerization of actin cables (Klemke et al., 1997). Thus, ERK1/2 signaling in focal adhesions can potentiate cell movement on the extracellular matrix.

The importance of the relationship between the ECM and ERK1/2 is evident in cancer. Activation of ERK1/2 is sufficient to transform NIH 3T3 cells (Mansour et al., 1994), and activating mutations in Ras account for at least 30% of all human cancers (Malumbres and Pellicer, 1998). In addition, the loss of ECM proteins such as fibronectin is correlated with a transformed phenotype (Hynes, 1992). A human fibrosarcoma cell line, designated HT1080, has an activated allele of Ras (Hall et al., 1983; Rasheed et al., 1974) and lacks assembly of a fibronectin matrix (Brenner et al., 2000). Matrix assembly can be restored in HT1080’s by blocking ERK1/2 activation with an inhibitor, or by stimulating integrin activation with Mn$^{2+}$ treatment or an activating antibody (Brenner et al., 2000). Although this example provides additional evidence that
ERK1/2 influences a cell’s interaction with the ECM, the exact mechanism of how ERK1/2 crosstalks with integrins to suppress matrix assembly is unknown.

**Integrin Signaling**

Integrin adhesion receptors are a family of heterodimeric transmembrane proteins expressed in all metazoans and consist of an α and β subunit that link the extra-cellular matrix (ECM) with the intra-cellular cytoskeleton. In addition to providing a physical link between cells and their environment, integrins transmit signals through this connection. These signals play central roles in adhesion, cell migration, differentiation, and proliferation (Hynes, 2002). Integrin activation is a term associated with a conformational change resulting in high-affinity ligand binding. The binding of ligands that transmit signals into the cell is referred to as outside-in signaling; whereas signals from within the cell that increase integrin-ligand binding affinity are referred to as inside-out signaling.

Outside-in integrin signaling functions to strengthen adhesion sites and regulate cell shape, cell polarity, and cell migration. In addition to these physical activities, outside-in signaling can regulate gene expression, cell proliferation, cell differentiation, and apoptosis. These intracellular signaling events mediated by integrins are similar to those activated by RTKs. However, rather than adaptor proteins or scaffold proteins to initiate the signaling process, outside-in signaling is dependent on Src family protein tyrosine kinases (SFKs) (Klinghoffer et al., 1999; Playford and Schaller, 2004). The importance of this type of signaling is perhaps best exemplified in platelets. Upon binding of the platelet integrins
αIIbβ3 to fibrinogen, c-Src becomes active (Obergfell et al., 2002; Arias-Salgado et al., 2003) and is necessary for cell spreading, second-wave platelet aggregation, and clot retraction (Shattil et al., 2004; Phillips et al., 2001). The activation of c-Src is followed shortly thereafter by activation of focal adhesion kinase (FAK) and a subsequent αIIbβ3 – c-Src-FAK complex (Shattil, 2005). This serves to promote changes in actin and microtubule structures and influence the activity of RHO-family GTPases (Mitra et al., 2005), thereby regulating adhesion and motility. In addition to regulating cell migration, the c-Src-FAK complex provides a docking site for the Grb2 adaptor protein, and promotes activation of ERK1/2 (Schlaepfer et al., 1999). This is evidence that both integrins and RTKs can activate the ERK1/2 MAP kinase cascade, and raises questions of how much ‘crosstalk’ exists between them. Can outside-in integrin signaling and RTKs synergize to promote prolonged and substantial ERK1/2 activation? Or is integrin induced ERK1/2 activation specific for events at focal adhesions?

The cytoplasmic tails of integrins, in particular a highly conserved NPxY motif on β integrins, are important for the binding of proteins to regulate inside-out integrin activation (Du et al., 1993; O’Toole et al., 1994; Calderwood et al., 1999). The final events leading to integrin activation culminate with talin binding to the β integrin cytoplasmic tail (Tadokoro et al., 2003; Tanentzapf et al., 2006). Thus talin is necessary and sufficient for integrin activation (Petrich et al., 2007; Ye et al., 2010). Recent studies have demonstrated that talin induces activation by binding first to the NPxY motif of β integrins via its phosphotyrosine binding
(PTB) domain (Calderwood et al., 2002). This docking site permits talin to then bind to a membrane proximal region of β integrins resulting in allosteric rearrangements of the αβ heterodimer causing an extracellular conformation change, and hence integrin activation (Wegener et al., 2007). Mutations in β integrins or talin that inhibit the interaction at the membrane proximal or distal site result in the loss of integrin activation (Wegener et al., 2007).

Other PTB domain-containing proteins can bind to β integrin cytoplasmic tails, but in contrast to talin, they negatively regulate integrin activation (Calderwood et al., 2003). Furthermore, phosphorylation of the NPxY motif of β integrin tails can function as a molecular switch to regulate integrin activation (Ling et al., 2005; Oxley et al., 2007). For example, phosphorylation of Y747 in the β3 integrin tail causes the DOK1 PTB domain to bind with a higher affinity than talin does. Structural studies of DOK1 reveal that two arginine residues form a pocket for negatively charged groups such as phospho-tyrosine (Oxley et al., 2007; Anthis et al., 2009). Moreover, this arginine pocket is conserved among all IRS-1 like PTB domains. This establishes a two step mechanism where the β tail is tyrosine phosphorylated, followed by the binding of a PTB containing protein(s), resulting in the loss of talin binding and the inactivation of integrins. Further insight has come from studies looking at non-phosphorylatable integrins with tyrosine to phenylalanine mutations. Fibroblasts expressing β1 Y783F/Y795F (diYF) exhibit impaired directed cell migration but increased fibronectin binding (Sakai et al., 1998). In addition, the diYF mutation causes slowed cell spreading and decreased FAK activity (Wennerberg et al., 2000).
Moreover, mice with the knock-in diYF mutation in β3 display a severe rebleeding defect (Law et al., 1999), although the β1 knock-in does not exhibit a dramatic phenotype (Chen et al., 2006; Czuchra et al., 2006). The relationship between DOK1, talin, and tyrosine phosphorylation of β integrin cytoplasmic tails is explored further in chapter 3.

FRS2α also contains an IRS-1 like PTB domain and has been previously described as a membrane linked docking protein involved in both upstream and downstream ERK1/2 MAP kinase signaling. Interestingly, the PTB domain of FRS2α binds to the juxtamembrane region of FGFRs independent of phosphorylation (Ong et al., 2000; Xu et al., 1998) whereas it binds to tyrosine-phosphorylated neurotrophin receptors (TRKs) and tyrosine kinase rearranged during transformation (RET) specifically at NPxpY motifs (Peng et al., 1995; Meakin et al., 1999; Melillo et al., 2001; Kurokawa et al., 2001). Interestingly, the PTB domain of FRS2α binds with a greater affinity to NPxpY motifs than to the juxtamembrane region of FGFRs (Yan et al., 2002) and growing evidence suggests that a sequence containing threonine residues phosphorylated by ERK1/2 adjacent to the FRS2α PTB domain acts as a molecular switch for FRS2α substrates (Yan et al., 2002; Zhou et al., 2009). In addition, previous work in our lab demonstrated that HRas, Raf-1 and ERK1/2 of the MAP kinase signaling pathway also inhibit integrin activation (Hughes et al., 1997).

Furthermore, ERK1/2 kinase activity is required for this function, and they exert their effects at the plasma membrane (Chou et al., 2003). The mechanism of how the ERK1/2 MAP kinase pathway exerts this function is unknown, and the
hypothesis that ERK1/2 phosphorylates FRS2α to inhibit integrin activation is explored further in chapter 4. Understanding how this occurs in cells will aide in understanding the multiple ways ERK1/2 can affect cell migration and adhesion.
REFERENCES


Chapter 2

Phosphoprotein enriched in astrocytes 15kDa (PEA-15) reprograms growth factor signaling by inhibiting threonine phosphorylation of fibroblast receptor substrate 2 alpha.

INTRODUCTION

Growth factor receptors transmit signals that regulate cell proliferation and differentiation, promote cell migration and survival, and modulate cellular metabolism. The mitogen activated protein (MAP) kinase pathway is an essential effector of a growth factor receptor signaling. The terminal elements of this pathway are the extracellular regulated kinases ERK1 and ERK2. ERK1/2 elicit biological outputs by phosphorylating nuclear targets such as the transcription factor Elk-1 (Gille et al., 1995), cytoplasmic substrates that include stathmin (Lovric et al., 1998) and RSK2 (Frodin et al., 1999), and membrane targets such as fibroblast receptor substrate 2 (FRS2α) (Lax et al., 2002). The localization of ERK1/2 dictates its access to substrates and therefore its biological activities. Proteins that regulate the localization of ERK1/2 include kinase suppressor of Ras (KSR), MEK-partner 1 (MP1), Sef, Paxillin, and phosphoprotein enriched in astrocytes (PEA-15) (Jacobs et al., 1999, Formstecher et al., 2001; Zhou 2002; Teis et al., 2002; Ishibe et al., 2003; Torii et al., 2004). PEA-15, in particular, functions as a potent inhibitor of ERK1/2 mediated transcription and cell proliferation by binding directly to ERK1/2 and preventing nuclear localization (Formstecher et al., 2001).
PEA-15 was originally discovered in astrocytes (Araujo et al., 1993) and subsequently found to be widely expressed in a variety of tissues and is conserved among mammals (Danziger et al., 1995). PEA-15 expression has been implicated in numerous pathologies including glioma, breast cancer, ovarian cancer, astrogliosis, and diabetes (Bera et al., 1994; Hwang et al., 1997; Condorelli et al., 1998; Tsukamoto et al., 2000; Embury et al., 2001; Underhill et al., 2001; Hao et al., 2001; Sharif et al., 2004; Glading et al., 2007; Bartholomeusz et al., 2008). PEA-15 comprises of an N-terminal death effector domain (DED) and a largely unstructured C-terminal tail (Hill et al., 2002). PEA-15 binds directly to ERK1/2, and limits ERK1/2 entry into the nucleus by blocking nuclear import and promoting nuclear export (Formstecher et al., 2001; Whitehurst et al., 2004). NMR “footprinting” and site-directed mutagenesis show that residues in the DED and in the tail of PEA-15 are involved in ERK1/2 binding (Formstecher et al., 2001; Hill et al., 2002). Furthermore, Protein Kinase C and CaMKII/AKT phosphorylate PEA-15 at Ser104 and Ser116 respectively, thus inhibiting ERK1/2 binding (Krueger et al., 2005). Whereas PEA-15 inhibits ERK1/2 phosphorylation of the nuclear transcription factor Elk-1, it does not inhibit the phosphorylation of ERK1/2 cytosolic targets such as stathmin or RSK2 (Formstecher et al., 2001; Vaidyanathan et al., 2003; Krueger et al., 2005). Therefore, PEA-15 functions to redirect ERK1/2 signaling rather than to inhibit ERK1/2 intrinsic kinase activity. Paradoxically, the expression of PEA-15 enhances activation of Ras and thus Raf-1 and MEK1/2 that lead to ERK1/2
phosphorylation and activation (Ramos et al., 2000). The mechanism by which PEA-15 potentiates the ERK1/2 MAP Kinase pathway is not known.

Here we define the mechanism whereby PEA-15 increases activation of ERK1/2. Structure-function analysis revealed that PEA-15 binding to ERK1/2 is required for activation of MEK1/2. PEA-15 blocked the association of ERK1/2 with the plasma membrane, thereby preventing threonine phosphorylation of FRS2α, a signaling adapter that links several tyrosine kinase growth factor receptors to Ras and ERK1/2 activation. Increased PEA-15 lead to reduced ERK1/2 dependent threonine phosphorylation and therefore to prolonged tyrosine phosphorylation of FRS2α, resulting in sustained activation of MEK1/2 and thus ERK1/2. Furthermore, the capacity of PEA-15 to sustain signaling downstream of FRS2 is the primary mechanism by which it activates the MAP kinase pathway because genetic deletion of FRS2α abrogated the capacity of PEA-15 to activate MEK1/2. Thus, PEA-15 can prolong upstream activation of growth factor signaling and the MAP kinase pathway, while preventing cell proliferation by blocking the transcriptional activities of ERK1/2. Expression of this protein therefore reprograms the output and duration of growth factor signaling.
MATERIALS AND METHODS

Plasmids

PEA-15 cDNA expression constructs used in this work have been described previously (Chou et al., 2003). The PEA-15 mutant L123R was described in Hill et al., (2002). Additional PEA-15 mutants S104D, S116D, and S104D S116D, were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by using wild-type pCDNA3-PEA-15 as the template. Human FRS2α cDNA was obtained from American Type Culture Collection (Manassas, VA) and subcloned into pCDNA3 via PCR. The FRS2α mutant T8V was generated with the QuickChange site-directed mutagenesis kit. All PEA-15 and FRS2α constructs in pCDNA3 included a C-terminal HA tag and were verified by DNA sequencing. A plasmid-based shRNA directed against PEA-15 and a control shRNA containing a scrambled sequence were constructed in pSilencer-U6 (Ambion, Austin, TX) and were a gift from Dr. Joseph Ramos (University of Hawai‘i-Manoa, Honolulu, HI) and have been previously described (Glading et al., 2007).

Cell Culture

Chinese Hamster Ovary (CHO)-K1 cells and glioblastoma A172 cells were obtained from the American Type Culture Collection (Manassas, VA). The generation of Raf-1:ER cells was previously described (Hughes et al., 1997). MEF cells carrying homozygous LoxP-flanked FRS2α alleles have been
previously described (Lin et al., 2007). All cells were cultured in DMEM (BioWhittaker, Walkersville, MD) containing 10% FCS, 1% nonessential amino acids, 2 mM glutamine (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin. Transient transfections of CHO, Raf-1:ER, and MEF cells were carried out with Lipofectamine Plus (Invitrogen, Carlsbad, CA) as described by the manufacturer. A172 cells were transfected using a nucleofection device (Lonza, Walkersville, MD) in combination with solution V and program U-029 (Lonza, Walkersville, MD).

**Antibodies and Reagents**

Anti-ERK2, -ERK1, -beta1 integrin, -Rho GDI, and -FRS2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2, -MEK1/2, -phospho-MEK1/2, -phospho-FRS2α (Tyr196), and -phospho-threonine-proline were obtained from Cell Signaling Technology (Danvers, MA). Anti-HA monoclonal antibody was purchased from Covance (Princeton, NJ). Rabbit polyclonal anti-PEA-15 (4513) was raised against a glutathione S-transferase-PEA-15 fusion protein (GST-PEA-15) as described previously (Krueger et al., 2005). Unless otherwise indicated, all antibodies were used at a 1:1000 dilution. 4-Hydroxy tamoxifen (4’OHT) was obtained from Sigma (St. Louis, MO) and used at a final concentration of 300 nM for the induction of Raf-1. Calf Intestinal Phosphatase (CIP) was purchased from NEB (Ipswich, MA) and incubated with cell lysates for 2 hours at room temperature.
Subcellular Fractionation

CHO cells were transfected with cDNA encoding wild-type PEA-15, PEA-15 mutant L123R, or empty vector. Cells were allowed to recover from transfection by allowing growth in complete media for 24 h. Next, cells were harvested in fractionation buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM NaF, 5 mM KCl, 1 mM NaVO₄, with Complete EDTA Free protease inhibitor cocktail [Roche Diagnostics]). After incubation on ice for 20 min, cells were homogenized by shearing through a 27-gauge needle. Forty microliters of total cell lysate was saved, and the remaining sample was centrifuged at 2000 rpm to sediment the nuclei. The supernatant was then spun at 14,000 rpm for 30 min at 4°C to separate the sedimented membrane fraction from the soluble cytosolic fraction. The membrane pellet was washed with 1 ml of lysis buffer, centrifuged at 14,000 rpm for 20 min at 4°C, and re-suspended in 180 µl of lysis buffer containing 1% NP-40. Total, cytosolic, and membrane fractions were resolved by SDS-PAGE and analyzed by Western blotting.
RESULTS

PEA-15 binding to ERK1/2 is necessary for PEA-15 stimulation of the ERK MAP kinase pathway.

Increased expression of PEA-15 activates Ras leading to activation of MEK1/2 and resulting activation of ERK1/2 (Ramos et al., 2000). PEA-15 binds to ERK1/2 (Formstecher et al., 2001); we therefore studied ERK1/2 binding-defective PEA-15 mutants to determine whether ERK1/2 binding was involved in activation of the MAP kinase pathway. PEA-15 binding to ERK1/2 is mediated by both the N-terminal death-effector domain (DED) and the C-terminal tail of PEA-15 (Figure 2.1A). In particular, mutation of PEA-15 Asp74 or Leu123 (Figure 2.1A) block ERK1/2 binding (Hill et al., 2002). Cells transfected with wild type or either the D74A or L123R mutant PEA-15 were monitored for phosphorylated MEK1/2 and phosphorylated ERK1/2 to assess the activation of the MAP kinase pathway. Expression of wild type PEA-15 resulted in the expected increase of phosphorylated MEK1/2 and ERK1/2 (Ramos et al., 2000), compared to cells transfected with an empty DNA vector. In contrast, neither cells transfected with the L123R nor D74A PEA-15 mutant increased MEK1/2 or ERK1/2 phosphorylation (Figure 2.1B) in spite of expressing similar levels of PEA-15. These data show that mutations in 2 distinct domains of PEA-15 that disrupt ERK1/2 binding abrogate PEA-15’s capacity to activate MEK1/2 and their downstream targets.
Protein Kinase C and CaMKII or AKT phosphorylate PEA-15 at Ser104 and Ser116 respectively, thus inhibiting ERK1/2 binding (Krueger et al., 2005). As an additional test of the role of ERK1/2 binding in activation of the MAP Kinase pathway, we examined the effect of Asp substitutions that mimic the effect of these Ser phosphorylations. PEA-15 mutants containing aspartic acid at either of the serine phosphorylation sites (S104D, S116D) or a mutant containing both mutations (S106,S116D) exhibited marked reduction in their capacity to stimulate phosphorylation of MEK1/2 and ERK1/2 (Figure 2.1C, 2.1S). Thus, 4 different mutations that disrupt ERK1/2 binding block PEA-15’s ability to activate the MAP kinase pathway. Notably the L123R mutation lies outside the region required for the binding of Phospholipase D (Zhang et al., 2000; Viparelli et al., 2008) and D74A, S104D, and S116D mutants do not affect binding to RSK2 (Vaidyanathan et al., 2003). Thus, we conclude that PEA-15 binding to ERK1/2 is required for PEA-15 mediated activation of the ERK1/2 MAP kinase pathway.
Figure 2.1: The binding of PEA-15 to ERK1/2 is required for activation of the ERK MAP kinase pathway.

A) Ribbon representation of the three dimensional structure of PEA-15 (Protein Data Bank accession code 1N3K) with helices colored in blue and the loops of the C-terminal in red. The side chains of selected residues are drawn as ball-and-stick models with Asp74 and Leu123 in yellow and Ser104 and Ser116 in green. B) Wild-type PEA-15 increases levels of endogenous pMEK1/2 and pERK1/2, whereas mutations in PEA-15 which abrogate binding to ERK1/2 (L123R and D74A) do not. CHO cells were transfected with cDNA’s encoding HA-tagged wild-type PEA-15 or the indicated PEA-15 mutants. After 24h, the cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting. The blots were quantified by densitometric scanning and represented in bar graphs below +/- SEM. C) Phosphomimetic mutant PEA15 (S104D, S116D, or double mutant S104D S116D) with reduced binding to ERK1/2, do not increase pMEK1/2 and pERK1/2 to the same extent as wild-type PEA-15.
A.

B.

C.
CHO cells were transfected with cDNA's encoding HA-tagged wild-type PEA-15 or the indicated PEA-15 mutants. After 24h, the cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting.

Figure 2.1S: Phosphomimetic mutations of PEA-15 do not increase pMEK1/2 and pERK1/2
PEA-15 blocks ERK1/2 membrane association.

Binding of PEA-15 to ERK1/2 re-routes ERK1/2 from the nucleus to the cytosol (Formstecher et al., 2001; Whitehurst et al., 2004; Krueger et al., 2005). Because the activation of Ras, the first step in the MAP kinase pathway, occurs at the cell membrane (Quilliam et al., 1995; Downward 1996), we examined the capacity of PEA-15 to inhibit ERK localization to the plasma membrane. We transfected CHO cells with cDNA encoding PEA-15 or ERK1/2 binding-defective PEA-15(L123R), and examined the localization of endogenous ERK2 in the membrane and cytosol. Expression of PEA-15 reduced membrane-associated ERK2 compared to PEA-15 (L123R) or an empty vector (Figure 2.2A). Similar results were observed for the localization of ERK1 (Figure 2.2S). Quantification revealed that PEA-15 caused a marked decrease in membrane-localized ERK2 and a corresponding increase in the cytosolic fraction (Figure 2.2B). In contrast, PEA-15 (L123R) had no effect on the distribution of ERK2 (Figure 2.2B). As expected, PEA-15 was confined to the cytosol (Danziger et al., 1995) as was the marker RhoGDI (Shimizu et al., 1991) (Figure 2.2A). Similarly, the membrane marker, β1 integrin was detected only in the membrane fraction, thereby confirming the subcellular fractionation. Thus PEA-15 sequesters ERK2 in the cytosol, limiting its association with the cell membrane (Figure 2.2C).
Figure 2.2: PEA-15 blocks the association of ERK1/2 with the plasma membrane.

CHO cells were transfected with cDNA’s encoding HA-tagged PEA-15, PEA-15 (L123R), or empty vector. After 24h, the cells were disrupted by osmotic shock, and fractionated into membrane and cytoplasmic fractions. Each fraction or the total lysate was separated by SDS-PAGE, and analyzed by immunoblotting. Levels of ERK2 were quantified by densitometric scanning. A) Wild-type PEA-15 reduced membrane association of ERK2, whereas the PEA-15 (L123R) did not. Beta 1 integrin and RHO GDI serve as validation of subcellular fractionation for membrane and cytoplasmic fractions respectively. B) Bar graph depicting the mean of data from 3 independent experiments +/- SEM. Wild-type PEA-15 decreases levels of endogenous ERK2 in membrane fractions, and increases levels of endogenous ERK2 in cytoplasmic fractions. The PEA-15 mutant L123R has little effect on ERK2 localization.
CHO cells were transfected with cDNA's encoding HA-tagged PEA-15, PEA-15 (L123R), or empty vector. After 24h, the cells were disrupted by osmotic shock, and fractionated into membrane and cytoplasmic fractions. Each fraction or the total lysate was separated by SDS-PAGE, and analyzed by immunoblotting. Wild-type PEA-15 reduced membrane association of ERK1/2, whereas the PEA-15 (L123R) did not. Beta1 integrin and RHO GDI serve as validation of subcellular fractionation for membrane and cytoplasmic fractions respectively.

Figure 2.2S: PEA-15 blocks the association of ERK1/2 with the plasma membrane.
PEA-15 blocks threonine phosphorylation of FRS2α.

As noted above, PEA-15 blocked ERK1/2 localization to the plasma membrane leading us to seek potential membrane-associated ERK1/2 substrates. The docking protein, FRS2α, is myristoylated resulting in membrane localization. ERK1/2 phosphorylates FRS2α, thereby inhibiting its tyrosine phosphorylation by growth factor receptors to reduce binding of Grb2 – SOS complex leading to reduced Ras activation, and ultimately ERK1/2 activation (Lax et al., 2002). We utilized a gel mobility shift to assay phosphorylation of FRS2α. Cells were transfected with cDNA encoding HA-tagged FRS2α, lysed, and anti-FRS2α immunoprecipitates were treated with an alkaline phosphatase or left untreated. Immunoblotting with an anti-HA or anti-phospho-threonine antibody revealed that the upper band detected by the HA antibody contained phosphorylated threonine and this band was eliminated by dephosphorylation with alkaline phosphatase (Figure 2.3A). This result confirms that the large mobility shift of FRS2α (Lax et al., 2002) can be used to monitor threonine phosphorylation. Over-expression of PEA-15 lead to a reduction in the upper band of FRS2α (Figure 2.3B, 2.3S), whereas, expression of PEA-15 (L123R) did not reduce threonine phosphorylated FRS2α (Figure 2.3C). We used a CHO cell line containing tamoxifen-regulated Raf-1 to activate ERK1/2 (Hughes et al., 1997) to increase threonine phosphorylation of FRS2α. When these cells were treated with tamoxifen, 64% of FRS2α was phosphorylated, whereas PEA-15-expressing cells exhibited a reduction in phosphorylation (41% phosphorylated) (Figure 2.3D). Importantly, in the absence of tamoxifen, the PEA-15 effect was
much stronger (Figure 2.4S), indicating that increased ERK activation can partially bypass the PEA-15 effect. Thus, PEA-15 blocks ERK1/2 mediated threonine phosphorylation of FRS2α (Figure 2.3E).
Figure 2.3: PEA-15 inhibits threonine phosphorylation of FRS2α.

A) The mobility shift of FRS2 is due to threonine phosphorylation. CHO cells were transfected with cDNA’s encoding HA-tagged FRS2α. After 24h, the cells were lysed and split into two fractions. One fraction was treated with calf intestinal alkaline phosphatase (CIP) at RT for 2h, while the other fraction was left untreated. Each fraction was immunoprecipitated with anti-FRS2, separated by SDS-PAGE, and immunoblotted with anti-HA or anti-pThr antibodies. B) PEA-15 reduces threonine phosphorylation of FRS2α. CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2α and HA-tagged PEA-15 in indicated samples. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Immunoblot of ERK2 serves as a loading control. Threonine phosphorylation of FRS2α was quantified by densitometric scanning and represented in bar graph below. C) A PEA-15 mutant that abrogates binding to ERK1/2 do not reduce threonine phosphorylation of FRS2α. CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2α in combination with either HA-tagged wild-type PEA-15 or PEA-15(123R). Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Note that PEA-15(123R) does not reduce the upper band of FRS2α to the same extent as wild-type PEA-15. Immunoblot of ERK2 serves as a loading control. D) Raf-ER CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2 in combination with either HA-tagged PEA-15 or empty vector. After 24h, the cells were treated with 4-hydroxytomoxifen (4-OHT) for 30m at 37°C. Cells were lysed immediately and analyzed by SDS-PAGE followed by immunoblotting. Note that the upper band of FRS2α is reduced in the presence of PEA-15. Immunoblot of pERK1/2 serves as a control for Raf-MEK-ERK activation following the addition of 4-OHT. Threonine phosphorylation of FRS2α was quantified by densitometric scanning and represented in bar graph below. E) PEA-15 inhibits ERK mediated threonine phosphorylation of FRS2α.
A. FRS2  +  +  +  CIP  -  +  +  pFRS2  FRS2  
   IB: FRS2
   IB: pThr

B. PEA-15  +  -  
   FRS2  
   ERK2  
   PEA-15  
   pFRS2  FRS2
   % of Threonine Phosphorylation

C. FRS2  +  +  -  +  
   PEA-15  -  +  +  -  
   PEA-15L123R  +  -  -  -  
   FRS2  
   ERK2  
   PEA-15  

D. 4-OHT  +  +  
   PEA-15  +  -  
   pFRS2  FRS2  

E. PEA-15  
   FRS2  
   ERK  
   % of Threonine Phosphorylation
CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2α and HA-tagged PEA-15 in indicated samples. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Note that only the upper band of FRS2α is detected by an antibody to phospho-threonine. No detectable threonine phosphorylation is present when PEA-15 and FRS2α are co-transfected.

![Figure 2.3S: PEA-15 inhibits threonine phosphorylation of FRS2α.](image)

Raf-ER CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2 in combination with either HA-tagged PEA-15, HA-tagged PEA-15 (L123R), or empty vector. After 24h, indicated samples were treated with 4-hydroxytomoxifen (4-OHT) for 60m at 37oC. Cells were lysed immediately and analyzed by SDS-PAGE followed by immunoblotting. Note that the upper band of FRS2α is enriched in PEA-15 samples treated with 4-OHT compared to untreated samples. Immunoblot of pERK1/2 serves as a control for Raf-MEK-ERK activation following the addition of 4-OHT.

![Figure 2.4S: An increase in active ERK1/2 can partially reverse PEA-15 inhibition of FRS2α threonine phosphorylation.](image)
Depletion of endogenous PEA-15 decreases tyrosine phosphorylation of FRS2α.

The foregoing experiments demonstrated that PEA-15 binding to ERK1/2 blocks their localization to the plasma membrane and reduces threonine phosphorylation of FRS2α. Such threonine phosphorylation is known to reduce FGF-induced tyrosine phosphorylation of FRS2α (Lax et al., 2002). To examine the role of endogenous PEA-15 in regulating FRS2α signaling, we used glioblastoma A172 cells that express PEA-15 (Glading et al., 2007). shRNA transfection induced a 90% reduction in PEA-15 expression (Figure 2.4A). When these cells were treated with FGFb, FRS2α tyrosine phosphorylation at residue 196 was markedly reduced in cells that had been depleted of PEA-15 (Figure 2.4A). Phosphorylation of tyrosine 196 functions as a docking site for Grb2-SOS complexes (Kouhara et al., 1997), and is therefore important for downstream MAP kinase signaling. We quantified three such experiments and observed statistically significant (p=.01) fivefold reduction in FGF-induced FRS2α tyrosine phosphorylation in PEA-15-depleted cells (Figure 2.4B) at 20 minutes after FGFb addition. By 40 min after FGFb addition, no increase in FRS2α tyrosine phosphorylation was observed in PEA-15-depleted cells. Thus, endogenous PEA-15 increases and prolongs FRS2α phosphorylation in response to FGFb.
A172 cells were transfected with shRNA for PEA-15 (shPEA15) or a scrambled shRNA (shCtrl). All plates were serum starved for 16h prior to stimulation with 100ng/ml FGFb for the indicated amount of time. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Levels of FRS2α tyrosine phosphorylation (pTyr FRS2) were quantified by densitometric scanning. A) Top panel indicates that tyrosine phosphorylation of FRS2α (pTyr FRS2) is abundant at 20 min and 40 min in shCtrl samples, whereas reduced pTyr FRS2 is observed at the same time points in shPEA15 samples. All samples were run on the same gel; irrelevant intervening bands were excised to save space. B) The bar graph summarizes the mean percent increase of pTyr FRS2 relative to the 0 time point from three independent experiments. Note that the knock down of PEA-15 greatly diminishes pTyr FRS2 in response to FGFb.

Figure 2.4: Knockdown of endogenous PEA-15 attenuates tyrosine phosphorylation of FRS2α.
PEA-15 prolongs FGF-induced activation of the ERK1/2 MAP kinase pathway.

Endogenous levels of PEA-15 affected the degree and duration of FGF-induced tyrosine phosphorylation of FRS2α, suggesting that it might regulate the duration of FGF-induced activation of the MAP kinase pathway. To test this idea, we over-expressed either PEA-15 or PEA-15(L123R) and examined the effect on FGF-induced FRS2α tyrosine phosphorylation and ERK2 phosphorylation. Over-expression of wild type PEA-15 increased tyrosine phosphorylation of FRS2α relative to over expression of PEA-15(L123R) (Figure 2.5 second row); conversely, the PEA-15(L123R)-transfected cells exhibited a more threonine phosphorylation of FRS2α than PEA-15-transfected cells, as indicated by the appearance of the FRS2α of reduced mobility (Figure 2.5 top row). The increased tyrosine phosphorylation of FRS2α in wild type PEA-15 transfected cells was associated with prolonged ERK2 activation (Figure 2.5 third row). In particular, PEA-15 over expression led to a markedly prolonged response at 60 minutes, a time when the PEA-15L123R cells exhibited maximal threonine phosphorylation (Figure 2.5 first row). Furthermore, PEA-15 did not increase ERK1/2 phosphorylation in cells expressing FRS2α T8V, an ERK phosphorylation-resistant mutant (Figure 2.5S). Thus, PEA-15 extends FGF-induced activation of ERK1/2 by reducing threonine phosphorylation leading to increased tyrosine phosphorylation of FRS2α.
Figure 2.5: Over-expression of PEA-15 prolongs tyrosine phosphorylation of FRS2α and phosphorylation of ERK1/2.

A) CHO cells were co-transfected with cDNA’s encoding HA-tagged FRS2α and either HA-tagged PEA-15 or PEA-15(L123R). All plates were serum starved for 16hr prior to stimulation of FGFb for the indicated length of time. Cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting with anti-HA (for the detection of FRS2α and PEA-15), anti-pY196 FRS2α, anti-pERK1/2, and anti-ERK2 antibodies. The kinetics of FGF-induced phosphorylation of FRS2α and ERK1/2 are altered in the presence of wild-type PEA-15 (left) relative to PEA-15(L123R) (right). Note that the upper band of FRS2α (previously established as threonine phosphorylated) is absent from cells transfected with wild type PEA-15, whereas a robust upper band of FRS2α in cells co-transfected with PEA-15 L123R appears at 60 and 120 min. The asterisks in the pERK1/2 indicate the prolonged pERK1/2 phosphorylation in the presence of wild-type PEA-15. B) Quantification of data (N=4) from experimental design described in Panel A. Depicted are the mean ± SEM the difference at 60 min was significant (p=0.003, T-test).
A

FGFb Treatment:
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PEA-15

PEA-15 L123R

B

Quantification of pERK1/2
CHO cells were co-transfected with cDNA's encoding HA-tagged FRS2α or a HA-tagged FRS2α mutant that does not contain threonine phosphorylation sites (T8V) in combination with either HA-tagged wild-type PEA-15 or PEA-15(L123R). Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Levels of pERK1/2 are similar in FRS2αT8V samples transfected with either PEA-15 or PEA-15(L123R).
FRS2α is required for PEA-15 to activate the MAP Kinase Pathway.

The previous studies established that PEA-15 binding to ERK1/2 blocked threonine phosphorylation of FRS2α, leading to prolonged tyrosine phosphorylation and activation of the MAP kinase pathway. We next asked if the capacity of PEA-15 to increase activation of this pathway was dependent on FRS2α. We utilized immortalized mouse embryo fibroblasts (MEF) derived from Frs2α flox mice and infected them with an adenovirus encoding Cre-recombinase resulting in the deletion of both Frs2α alleles (Frs2α-/- cells). As a control, cells were infected with adenovirus encoding LacZ. The two resulting MEF cell lines were transfected with PEA-15, PEA-15(L123R), or empty vector. MEF cells lacking FRS2α did not exhibit activation of MEK1/2 (left panel Figure 2.6A) when expressing wild-type PEA-15, whereas the control, lacZ adenovirus-infected cells did so (right panel Figure 2.6A). Re-expression of FRS2α in Frs2α-/- cells restored the phosphorylation of MEK1/2 in response to PEA-15 over expression (left panel Figure 2.6B). Thus FRS2α is required for PEA-15 to induce activation of the MAP Kinase pathway (Figure 2.6C).
Figure 2.6: FRS2α is required for PEA-15 to activate MEK1/2. FRS2α floxed MEF’s were transduced with an adenovirus encoding cre-recombinase or GFP.

After 48h, cells were transfected with cDNA’s encoding HA-tagged wild-type PEA-15, PEA-15(123R), and/or HA-tagged FRS2α. 24h post-transfection, cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting. A) PEA-15 does not increase MEK1/2 phosphorylation in cells deficient in FRS2α. Asterisk denotes the band corresponding to pMEK1/2. B) Re-expression of FRS2α restores PEA-15’s ability to activate MEK1/2. Asterisk denotes the band corresponding to pMEK1/2. C) Quantification of the data from four independent experiments described in panels A and B. Percent increase is calculated relative to cells transfected with empty vector +/- SEM.
DISCUSSION

The ERK1/2 MAP kinase pathway is a central regulator of cellular behaviors and PEA-15, an ERK1/2 binding protein, has profound effects on the activation and output of this pathway (Ramos et al., 2000; Formstecher et al., 2001). Here we have defined the mechanism whereby PEA-15 increases activation of MEK1/2 and consequently, ERK1/2 (Figure 2.7). Mutational analysis revealed that PEA-15 binding to ERK1/2 is required for activation of MEK1/2 and ERK1/2. Because this pathway is initiated by activated Ras at the plasma membrane, we examined the effects of PEA-15 on ERK1/2 localization and function at the membrane. PEA-15 blocked the association of ERK1/2 with the membrane, thereby preventing threonine phosphorylation of FRS2α, a membrane-tethered adapter that links several tyrosine kinase growth factor receptors to Ras and ERK1/2 activation. This threonine phosphorylation terminates FRS2α signaling by inhibiting tyrosine phosphorylation, thereby preventing the binding of downstream adapters such as Grb2 (Lax et al., 2002).

We now find that increased PEA-15 leads to prolonged tyrosine phosphorylation of FRS2α, which results in activation of MEK1/2 and thus ERK1/2. Furthermore, shRNA-mediated depletion of endogenous PEA-15 decreased FGF-induced tyrosine phosphorylation of endogenous FRS2α thereby establishing the biological relevance of PEA-15 regulation of this pathway. Importantly, these studies employed mouse embryo fibroblasts, human glioblastoma cells, and CHO cells, suggesting that these findings can be generalized to many cells.
types. Consequently, PEA-15 increases activation of the ERK 1/2 MAP Kinase pathway by interrupting a negative feedback loop formed by ERK1/2 phosphorylation of FRS2α. Finally, genetic deletion of FRS2α abrogated the capacity of PEA-15 to activate MEK1/2, establishing that this is the major mechanism whereby PEA-15 activates the MAP kinase pathway. Thus, we describe a novel mechanism for prolonging upstream activation of growth factor signaling and the MAP kinase pathway; in particular, PEA-15 binds ERK1/2 preventing its localization to the plasma membrane thereby inhibiting, threonine phosphorylation of FRS2α.

PEA-15 must bind to ERK1/2 to activate MEK1/2 and ERK1/2. Mutations in the folded PEA-15 DED (D74A) or its unstructured C-terminus (L123R) and phosphorylation-mimicking Asp mutations at Ser104 and Ser116 block ERK1/2 binding. Each of these mutations abrogated PEA-15’s capacity to activate MEK1/2 and ERK1/2. Importantly, these mutants were well expressed and several of them have only localized effects on the HSQC nuclear magnetic resonance spectrum of PEA-15 (Hill et al., 2002), indicating that these mutants do not disrupt the overall structure of PEA15. Furthermore, PEA-15 can bind to RSK2, however the D74A mutation does not impair this interaction (Vaidyanathan et al., 2003). Similarly, Leu 123 lies outside the region of PEA-15 that binds to Phospholipase D (Zhang et al., 2000; Viparelli et al., 2008). Yet each of these mutants disrupted activation of the MAP kinase pathway, indicating that binding of RSK2 or phospholipase D is not required for PEA-15's effect on MEK1/2 and ERK1/2 activation. Importantly, phosphorylation of PEA-15 acts as
a molecular switch that disengages PEA-15 from ERK1/2 (Krueger et al., 2005; Renganathan et al., 2005), and leads to PEA-15 recruitment to the death inducing signaling complex (DISC) (Condorelli et al., 1999; Kitsberg et al., 1999; Xiao et al., 2002). Our finding that PEA-15 phosphomimetic mutations block its ability to activate the MAP kinase pathway establish another regulatory role for PEA-15 phosphorylation.

We found a reduction of ERK2 in the membrane fraction of PEA-15 expressing cells and ascribe this effect to the interaction of PEA-15 with ERK1/2 because it was abolished by a L123R mutation that inhibits ERK binding. Previous studies showed that PEA-15 can reduce nuclear accumulation of ERK1/2 by both blocking nuclear entry (Whitehurst et al., 2004) and inducing nuclear export (Formstecher et al., 2001) thereby inhibiting the transcriptional effects of ERK 1/2. ERK1/2 are targeted to the plasma membrane by interacting with the scaffolding protein, KSR, that binds to membranes via its C1 domain (Muller et al., 2001; Zhou et al., 2002) leading to the formation of a Ras/MEK/ERK complex at the plasma membrane (Morrison et al., 2003). PEA-15 binds to ERK2 near the F-recruitment site (αG helix of ERK2) and ERK2 mutations at this site abrogate binding to PEA-15 (Chou et al., 2003) this same region is involved in binding to KSR (Jacobs et al., 1999; Cacace et al., 1999). Furthermore, PEA-15 blocks binding of ERK1/2 substrates (e.g. Elk-1) to the D-recruitment site (Callaway et al., 2007); an effect that may also contribute to blockade of membrane localization. In either case, PEA-15 blocks ERK1/2 signaling in the nucleus, and we now show that it blocks the membrane
localization of ERK2 and the phosphorylation of a membrane-associated substrate.

PEA-15 blocks ERK-mediated threonine phosphorylation of FRS2α, a membrane-tethered (Kouhara et al., 1997) ERK1/2 substrate (Lax et al., 2002; Wu et al., 2003), thereby prolonging its tyrosine phosphorylation and downstream signaling. FRS2α is similar to other adaptors proteins such as DOK, Gab, and IRS; however, the FRS2 family is unusual in that it is myristoylated and therefore constitutively membrane-associated. Thus, our finding that PEA-15 blocks membrane-recruitment of ERK1/2 suggests that FRS2α is a preferred target of PEA-15’s effects, an idea supported by the observation that deletion of FRS2α blocked the ability of PEA-15 to activate MEK 1/2; hence regulation FRS2α is a principal mechanism by which PEA-15 increases activation of the ERK1/2 MAP kinase pathway.

Our studies establish a mechanism whereby PEA-15 promotes and sustain growth factor signaling: by binding to ERK1/2, PEA-15 blocks ERK1/2 mediated phosphorylation of FRS2α, thereby interrupting a major negative feedback loop that terminates growth factor signaling. These results have important implications for the biological effects of PEA-15 expression. In addition to increasing ERK1/2 activation (Ramos et al., 2000) our data show that PEA-15 prolongs signaling downstream of FRS2α, suggesting much broader activating effects on signal transduction pathways. Unphosphorylated PEA-15 prevents nuclear translocation of ERK1/2 thereby inhibiting transcriptional effects of ERK1/2 that lead to cell cycle progression and tumor cell invasion (Formstecher
et al., 2001; Renault et al., 2003; Glading et al., 2007). Furthermore, PEA-15 can block TRAIL-induced apoptosis (Condorelli et al., 1999; Kitsberg et al., 1999; Xiao et al., 2002), increase expression of Phospholipase D (Zhang et al., 2000) to promote glucose transport (Viparelli et al., 2008), and act as a scaffold to promote activation of RSK2 (Vaidyanathan et al., 2007). This combination of activities suggest that high level expression of PEA-15 may enable non-proliferative cells to survive for extended periods. Notably, PEA-15 is expressed in astrocytes (Araujo et al., 1993) that can give rise to neural stem cells, a cell population that is long-lived yet minimally proliferative (Emsley et al., 2005).

Similarly, it is highly expressed in mammary epithelium and in some mammary cancers (Glading et al., 2007), a tumor type that is prone to prolonged survival of micrometastases (Demicheli et al., 1996). Our studies have elucidated a specific biochemical mechanism whereby PEA-15 prolongs growth factor signaling, thereby activating the MAP kinase pathway, thus contributing to its extensive armamentarium of biological effects.

Chapter 2, in full, is a reprint of the material as it appears in Molecular Biology of the Cell, 21 (4), 664-73 (2010). Haling, Jacob R; Wang, Fen; Ginsberg, Mark H. Jacob Haling was the primary investigator and author of this paper.
PEA-15 binds directly to ERK1/2 preventing its membrane localization. Consequently, this limits ERK-mediated threonine phosphorylation of the membrane tethered substrate FRS2α, resulting in increased and prolonged tyrosine phosphorylation and activation of downstream signaling pathways such as that leading to activation of ERK1/2 MAP kinase.

Figure 2.7: Schematic diagram of how PEA-15 inhibits a negative feedback loop between ERK1/2 and FRS2α.
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Chapter 3

Beta integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation.

INTRODUCTION

Integrins play a fundamental role in cell adhesion and migration, linking the extracellular matrix to the actin cytoskeleton. In the adult, integrins are essential for a variety of biological processes including wound healing, leukocyte trafficking, and angiogenesis, and are thus increasingly attractive therapeutic targets for a variety of conditions, notably cancer. Mammals express 18 different $\alpha$ subunits and 8 different $\beta$ subunits, which form 24 unique $\alpha$$\beta$ heterodimers (excluding splice variants). Each $\alpha$ and $\beta$ chain of the integrin heterodimer consists of several linked globular extracellular domains, a single membrane-spanning domain, and a short cytoplasmic tail (Hynes, 2002). The cytoplasmic tails of the $\beta$ subunits exhibit conserved regions (Figure 3.1A), and they act as a hub for protein-protein interactions (Lie et al., 2000), modulating a variety of signaling processes in both directions across the membrane. Extracellular adhesion can be activated from within the cell by the cytoskeletal protein talin (Hynes, 2002; Ginsberg et al., 2005; Campbell and Ginsberg, 2004; Calderwood, 2004) via a direct interaction between the talin F3 domain and the membrane-proximal (MP) portion of the integrin tail (Calderwood et al., 2002; Tadokoro et al., 2003; Wegener et al., 2007).
The different $\beta$ integrins found in mammals occupy unique biological niches (Hynes, 2002) and show significant diversity in their protein-protein interactions. Of the six $\beta$ integrins tails that display a high level of sequence homology to one another ($\beta_1$, $\beta_2$, $\beta_3$, $\beta_5$, $\beta_6$, and $\beta_7$), all contain two NPxY or NPxY-like motifs—a near membrane-distal (nMD) site and a far membrane-distal (fMD) site—that bind to phosphotyrosine-binding (PTB) domains (including the talin F3 domain) (Calderwood et al., 2003) and are potential phosphorylation sites. In the $\beta_2$ tail, however, both tyrosine residues are substituted with phenylalanine, and in the $\beta_7$ tail this substitution occurs in the fMD site. Uniquely, the $\beta_7$ tail also exhibits two additional MP tyrosine phosphorylation sites that are not part of an NPxY motif (Krissansen et al., 2006).

This study explores three of these integrins: $\beta_3$, $\beta_1A$ (the most common splice variant of $\beta_1$), and $\beta_7$ (Figure 3.1A). Previous studies on integrin tyrosine phosphorylation have generally focused on two of these: $\beta_3$ and $\beta_1$. When the $\beta_1$ integrin was first isolated and sequenced, a potential tyrosine phosphorylation site was proposed (Tamkun et al., 1986), and an early study observed $\beta_1$ tyrosine phosphorylation in response to transformation of cells with viral Src (v-Src) (Hirst et al., 1986), followed by the demonstration of tyrosine phosphorylation of $\beta_1$ by v-Src in vitro (Tapley et al., 1989). These studies involved a viral protein, but it was later found that $\alpha_{IIb}\beta_3$ is tyrosine-phosphorylated in response to platelet activation, and that cellular Src (c-Src) and related tyrosine kinases phosphorylate $\beta_3$ in vitro (Law et al., 1996). Studies suggest that $\beta_1$ interacts with various Src family kinases and that these kinases
are activated by integrin engagement with the extracellular matrix (Miller et al., 1999; Ulanova et al., 2005; Arias-Salgado et al., 2005; Arias-Salgado et al., 2003; Klinghoffer et al., 1999; Lowell, 2004; Hood et al., 2003; Huveneers et al., 2008). There is also evidence for a direct and constitutive interaction between β3 and c-Src (Shattil, 2005).

Various studies have demonstrated that tyrosine phosphorylation of β1 influences integrin localization and activity, as well as cell morphology. An early study showed that transformation of cells with Rous sarcoma virus—which expresses v-Src—leads to β1 integrins adopting a more diffuse distribution on the cell surface (Hirst et al., 1986), rather than being localized in focal adhesions—which are large macromolecular complexes composed of integrins and intracellular proteins, such as vinculin, talin, and paxillin, that serve as sites of tight attachment to the extracellular matrix (Burridge et al., 1996; Clark and Brugge, 1995). Transformed cells also display rounding, decreased fibronectin matrix assembly, and decreased cell migration (Hirst et al., 1986). Another study found that while unphosphorylated β1 integrins localize to focal adhesions, phosphorylated integrins localize to podosomes (Johansson et al., 1994).

Further insight has come from studies using non-phosphorylatable integrins with Y-to-F mutations. The Y783F mutation in the β1 nMD site reverses the effects of v-Src (Sakai et al., 2001). Fibroblasts expressing β1 Y783F, Y795F, or YY783/795FF display impaired directed cell migration but increased fibronectin binding (Sakai et al., 1998), and YY783/795FF also causes slowed cell spreading and decreased focal adhesion kinase activation (Wennerberg et
In β3, the nMD mutation Y747F disrupts adhesion and clot retraction in hematopoietic cells (Blystone et al., 1997). More tellingly, mice with the knock-in double YY747/759FF mutation in β3 display a severe bleeding defect (Law et al., 1999), although, surprisingly, mice with the analogous β1 knock-in mutation show no significant phenotype (Chen et al., 2006; Czuchra et al., 2006).

The role of tyrosine phosphorylation in outside-in β3 signaling is relatively well-established (Blystone et al., 1997; Law et al., 1999; Chandhoke et al., 2004; Gao et al., 2005; Butler and Blystone, 2005; Prasad et al., 2003; Cowan et al., 2000; Kirk et al., 2000; Jenkins et al., 1998; Xi et al., 2006). The role in inside-out integrin activation is less clear, but various lines of evidence indicate that phosphorylation negatively regulates activation. Tyrosine phosphorylation of αVβ3 decreases the affinity of live cells for fibronectin (Datta et al., 2002), and phosphorylation of β1 decreases its affinity for fibronectin in vitro (Tapley et al., 1989).

Dok1 is a signaling protein with a PTB domain capable of binding integrins (Calderwood et al., 2003). Dok1 negatively regulates β3 integrin activation (Wegener et al., 2007), an observation initially difficult to explain due to the very weak interaction observed between these proteins. We subsequently reported that tyrosine phosphorylation greatly increases Dok1 affinity for short β3 peptides while slightly decreasing talin1 affinity, observations that led to an initial structural explanation for this phenomenon (Figure 3.2) (Oxley et al., 2008). However, these findings did not clarify the specific roles of the different NPxY motifs or indicate whether this mechanism could be generalized across different integrins.
Here, we explore the phosphorylation dependence of the talin1 and Dok1 interactions with full-length β3, β1A, and β7 tails. We show that tyrosine phosphorylation is a common mechanism for regulating the affinity of these proteins for integrin tails, and a recent crystal structure of a talin/β1 complex (Anthis et al., 2009) allows us to explain subtle differences between different integrins. We also describe the structural basis of this phosphorylation state specificity in detail and generate a talin mutant that shows preferential binding for phosphorylated integrin tails.
(A) Sequences of the cytoplasmic regions of the β3, β1A, and β7 integrin tails. The two NPxY motif tyrosine residues are indicated, with β3 numbering. The membrane-proximal (MP), near membrane-distal (nMD), and far membrane-distal (fMD) regions are denoted. Secondary structure is based on the β1D/talin2 complex structure (Anthis et al., 2009), with α helices denoted in blue and 3¹ helices in green. (B) Sequences of the PTB domains of Dok1, talin1, and talin2 aligned by secondary structure, with secondary structure elements from the Dok1 PTB domain structure (PDB 2V76) (Oxley et al., 2008) shown. Notable residues are indicated with Dok1 numbering.
Figure 3.2: The structural basis of Dok1 specificity for phosphorylated integrin tails.

(A) Detail of the Dok1 PTB domain structure (PDB 2V76) (Oxley et al., 2008) showing a sulfate anion located in the NPxY binding pocket. Key positively-charged residues are highlighted. (B) Detail of the NPxY motif of the β3 integrin tail bound to the talin1 F3 domain (PDB 1MK9) (Garcia-Alvarez et al., 2003). (C) Detail of the NPxY motif of the β1D tail bound to the talin2 F3 domain (PDB 3G9W) (Anthis et al., 2009). The residues highlighted in panels B and C are analogous to those highlighted in the Dok1 structure in panel A. Molecular images were generated with MOLMOL (Koradi et al., 1996).
MATERIALS AND METHODS

Expression and purification of proteins

Proteins and peptides were expressed in *E. coli* and purified as reported previously (Anthis *et al.*, 2009) unless otherwise indicated. Integrin tail constructs corresponding to the entire predicted cytoplasmic region were produced in pET16b using the following boundaries: β3 K716-T762, β1A K752-K798, and β7 R747-L798. The talin1 F3 domain (G309-S405) and the Dok1 PTB domain (Q154–G256) were produced in pGEX-6P-2 as reported previously (Wegener *et al.*, 2007). For experiments in mammalian cells, the cDNA encoding full-length mouse talin1 was amplified by PCR and subcloned into pEGFP-C1. Mutations in pET and pGEX vectors were introduced with the QuikChange kit, and mutations in pEGFP-C1 were introduced with the QuikChange II XL kit (Stratagene).

Cell Culture

SYF cells (mouse embryonic fibroblasts (MEFs) deficient in c-Src, c-Fyn, and c-Yes) and SYF + Src cells (SYF MEFs reconstituted with c-Src) (Klinghoffer *et al.*, 1999) were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics at 37°C with 6% CO₂. Transient transfections were carried out with Lipofectamine Plus (Invitrogen) as described by the manufacturer.
Tyrosine phosphorylation of integrin tails

The kinase domain of c-Src (Q251-L533) in pET28 was co-expressed with *Yersinia pseudotuberculosis* YopH in pCDFDuet-1 and purified by immobilized metal affinity chromatography as previously reported (Seeliger *et al.*, 2005). Tyrosine phosphorylation was performed overnight at 30°C with 20 μM integrin tail and 0.015 mg/mL Src in 50 mM Tris, 20 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM ATP, 1 mM DTT, pH 7.0. Phosphorylated tails were separated from unphosphorylated tails by C$_4$ reverse phase HPLC and were identified by mass spectrometry and NMR. Preliminary experiments observed phosphorylation by autoradiography.

NMR spectroscopy

All NMR experiments were performed on spectrometers equipped with Oxford Instruments superconducting magnets (500, 600, 750, and 950 MHz $^1$H operating frequencies) and GE/Omega computers. Unless otherwise indicated, samples were prepared in NMR buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM DTT, pH 6.1) with 5% D$_2$O and Complete protease inhibitors (Roche). Experiments were performed at 25°C. The $^1$H and $^{15}$N resonances of $^{15}$N-labelled β7 integrin tail were assigned using a 0.2 mM sample in 20 mM sodium acetate pH 4.5 and employing 3D NOESY-HSQC and 3D TOCSY-HSQC spectra. Resonance assignments were transferred to pH 6.1 conditions through pH titrations. Spectra were referenced in the direct dimension to DSS at 0 ppm, with indirect referencing in the $^{15}$N dimension using a $^{15}$N/$^1$H frequency ratio of
0.101329118 (Wishart et al., 1995). Data were processed using NMRPipe (Delaglio et al., 1995) and spectra were visualized using the program SPARKY (www.cgl.ucsf.edu/home/sparky) or CCPN Analysis (Vranken et al., 2005). The $^1$H and $^{15}$N resonance assignments of the β7 tail have been deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) with the accession number 16259. Resonances of β3 and β1A integrin tails were previously assigned and deposited under accession numbers 15552 (Oxley et al., 2008) and 16159 (Anthis et al., 2009), respectively.

**Protein-Protein Interaction Studies**

$^1$H-$^{15}$N HSQC titrations (Supplementary Fig. 4) were performed with 0.05 mM $^{15}$N-labelled integrin tail and increasing concentrations of unlabelled talin1 or Dok1, from 0 to 1 mM. Weighted combined $^1$H and $^{15}$N amide shifts ($\Delta$(H,N)) were calculated using the equation:

$$\Delta(H,N) = \sqrt{\Delta_H W_H^2 + \Delta_N W_N^2}$$

where $W_H$ and $W_N$ are weighting factors for the $^1$H and $^{15}$N amide shifts, respectively ($W_H = 1$, $W_N = 0.154$) (Ayed et al., 2001) and $\Delta = \delta_{\text{bound}} - \delta_{\text{free}}$.

Dissociation constants were determined by fitting changes in backbone chemical shifts upon increasing talin concentration to the following equation:

$$\Delta(H,N) = \Delta(H,N)_{\text{max}} \left[ (L) + [U] + K_d - \sqrt{((L) + [U] + K_d)^2 - 4(L)[U])} \right] / 2[L]$$

where $K_d$ is the dissociation constant, $\Delta(H,N)_{\text{max}}$ is the shift change at saturation, $\Delta(H,N)$ is the weighted shift change, and [L] and [U] are the concentrations of the labelled and unlabelled proteins,
respectively. Data from peaks that were well-resolved, had a significant change in position, and were discernable throughout the titration were fit simultaneously to this equation with the program OriginPro 8, extracting a single $K_d$ and multiple $\Delta(H,N)_{\text{max}}$ values. Values for $\Delta G$ were calculated from the $K_d$ value.

Data are presented throughout the paper as the $K_d$ value ± standard error. This error only takes into account the uncertainty from the fitting procedure. The other source of error in these experiments would be concentration errors, but they are not reported due to difficulty in estimating them. However, experience indicates that errors in $K_d$ stemming from talin1 or Dok1 concentration determination would at most be about 10% (corresponding to a maximum error in $\Delta G$ of 0.25 kJ/mol). For interactions with $K_d$ values less than about 100 $\mu$M, this would be compounded by $\beta$ integrin concentration determination errors, leading to a maximum total $K_d$ error due to concentration errors of approximately 20% (corresponding to a maximum error in $\Delta G$ of 0.5 kJ/mol).

Some $K_d$ values are reported as approximate because binding was too weak for the generation of a binding curve. In these cases, the value of $\Delta(H,N)_{\text{max}}$ was estimated by comparing maximum $\Delta(H,N)$ values for relevant peaks to the $\Delta(H,N)$ of peaks in a corresponding quantifiable titration. The fitting procedure was then carried out as before, but with the value of $\Delta(H,N)_{\text{max}}$ restrained. No errors are reported for these values, as they are only estimates.
**Immunofluorescence imaging**

After transfection, MEF cells were plated on 7.5 μg/ml fibronectin-coated coverslips, allowed to adhere for 90 minutes in Dulbecco’s modified Eagle’s medium, rinsed once in phosphate-buffered saline (PBS), and fixed with 3.7% formaldehyde in PBS. After fixation, cells were permeabilized in 0.1% Triton X-100 for 5 minutes, blocked with 3% BSA, 2% normal goat serum for 1 hour, and then incubated with the appropriate primary antibody in blocking solution overnight at 4°C. Bound antibodies were detected by the corresponding fluorescein isothiocyanate-conjugated goat secondary antibodies (Santa Cruz Biotechnology). Coverslips were subsequently mounted in Prolong Gold antifade reagent (Invitrogen) on slides. Epifluorescent images of cells were acquired with a 60x oil immersion objective on a Nikon Eclipse TE2000-U microscope equipped with the appropriate excitation and emission filter sets (Semrock). Additional post-acquisition processing of images was performed using ImageJ (rsb.info.nih.gov/ij) and Adobe Photoshop.

Anti-green fluorescent protein (GFP) rabbit polyclonal antibody was obtained from Clontech. Anti-vinculin mouse monoclonal antibody was purchased from Sigma. Anti-phospho-tyrosine (pY100) mouse monoclonal antibody was purchased from Cell Signaling Technology. Anti-paxillin rabbit polyclonal antibody (RB4536) was developed in house.
RESULTS

Production of tyrosine-phosphorylated integrin tails for NMR.

Before the current study, structural work on integrin phosphorylation had involved short chemically-synthesized integrin tail fragment peptides (Oxley et al., 2008). However, we recently reported a robust system for studying integrin tail protein-protein interactions by NMR using full-length $^{15}$N-labelled integrin tails produced in *E. coli*. This system is cost-effective and versatile, but using such a system to produce tyrosine-phosphorylated peptides presents additional difficulties; modified residues can be incorporated directly during chemical peptide synthesis, but not in *E. coli*. Although glutamate can be introduced by mutagenesis to make an acceptable mimic for phosphoserine or phosphothreonine, phosphotyrosine has no natural analogue. Thus, the integrin tails would have to be phosphorylated directly.

After exploring other options, we were able to produce NMR-scale quantities of tyrosine-phosphorylated $^{15}$N-labelled integrin tails by *in vitro* phosphorylation with c-Src, which has been used for *in vitro* phosphorylation of integrin tails in previous studies (Law et al., 1996; Kirk et al., 2000), although not previously on a scale large enough for structural biology. Since tyrosine kinases can be toxic to bacteria, we produced the Src kinase domain in *E. coli* by coexpressing it with YopH phosphatase, as previously described by Seeliger et al. (2005).
In order to phosphorylate specific tyrosine residues selectively, single and double Y-to-F mutants were made: \(\beta_3\) Y747F (for pY759) and Y759F (for pY747), \(\beta_1A\) Y783F (for pY795) and Y795 (for pY783), and \(\beta_7\) YY753/758FF (for pY778). The \(\beta_7\) tail contains two MP tyrosine residues, but phosphorylation of just the nMD tyrosine residue was explored (the fMD site contains a natural Y-to-F substitution). Tyrosine phosphorylation caused localized perturbations in the HSQC spectra of the integrin tails (Figure 3.3). Phosphorylation of both \(\beta_3\) Y747F and Y759F was observed, as was phosphorylation of \(\beta_1A\) Y795F and \(\beta_7\) YY753/758FF. However, phosphorylation of \(\beta_1A\) Y783F was not observed. When the phosphorylation reaction was performed on \(\beta_1A\) wild type (WT), chemical shift perturbations were only observed near Y783, indicating that Y783, but not Y795, was phosphorylated in this system. Thus, for further studies, \(\beta_1A\) pY783 was produced from WT peptides.
Figure 3.3: Phosphorylation of integrin tails.

HSQC spectra of integrin tails before (red) and after (blue) the tyrosine phosphorylation reaction. Phosphorylated residues are indicated. (A) \( \beta 3 \) Y759F. (B) \( \beta 3 \) Y747F. (C) \( \beta 1A \) Y795F. (D) \( \beta 1A \) Y783F. (E) \( \beta 1A \) WT. (F) \( \beta 7 \) YY753/758FF.
Tyrosine phosphorylation decreases integrin affinity for talin.

Binding of proteins to integrin tails was assayed by observing chemical shift perturbations in integrin tail HSQC spectra. Upon the addition of the talin1 F3 domain to the β3, β1A, or β7 tail, significant perturbations were observed in the MP and nMD portions of the tail (Figure 3.4). The MP perturbations were greatest in the β3 tail, but they were present in all tails tested. The affinities of these interactions were quantified, giving $K_d$ values that ranged from 142 μM for β7 to 273 μM for β3 to 491 μM for β1A (Table 3.1). Smaller shifts were also observed in the fMD portion of the β3 integrin tail (and to a lesser extent in the β1A tail), but these perturbations are likely due to a weak competing integrin/talin interaction, with a $K_d$ of several mM for the fMD portion.

The introduction of Y-to-F mutations employed for specific phosphorylation in β3 and β7 had negligible effects on talin affinity in these integrins. The greatest effect was seen with Y747F in β3, which increased the $K_d$ to 366 μM (Table 3.1). Such a decrease in affinity upon a mutation in the talin binding site is not surprising, and it is notable that this effect is very small compared to the much greater changes in affinity observed elsewhere in this study.

Tyrosine phosphorylation of the nMD NPxY motif decreased the magnitude of chemical shift perturbations observed upon talin binding (Figure 3.4) and decreased the affinity of these interactions. This effect was most pronounced in β7, less so in β1A, and least in β3. Phosphorylation of the fMD NPxY motif in β3 abrogated chemical shift perturbations upon talin binding in that
region, but had little effect on the much tighter interaction with the MP and nMD regions or the overall affinity (Table 3.1).

Table 3.1: Effect of tyrosine phosphorylation on the affinity of the integrin/talin1 interaction

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$K_d$ (μM)$^a$</th>
<th>$\Delta G$ (kJ/mol)$^b$</th>
<th>$\Delta \Delta G_{pY}$ (kJ/mol)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>273 $\pm$ 6.4</td>
<td>-20.33 $\pm$ 0.06</td>
<td>-</td>
</tr>
<tr>
<td>Y759F</td>
<td>286 $\pm$ 4.6</td>
<td>-20.22 $\pm$ 0.04</td>
<td>-</td>
</tr>
<tr>
<td>Y759F pY747</td>
<td>1032 $\pm$ 27</td>
<td>-17.04 $\pm$ 0.07</td>
<td>3.21</td>
</tr>
<tr>
<td>Y747F</td>
<td>366 $\pm$ 6.4</td>
<td>-19.61 $\pm$ 0.04</td>
<td>-</td>
</tr>
<tr>
<td>Y747F pY759</td>
<td>386 $\pm$ 15</td>
<td>-19.48 $\pm$ 0.10</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>β1A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>491 $\pm$ 10</td>
<td>-18.88 $\pm$ 0.05</td>
<td>-</td>
</tr>
<tr>
<td>pY783</td>
<td>2,500 est.$^d$</td>
<td>-14.8</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>β7</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>142 $\pm$ 3.0</td>
<td>-21.95 $\pm$ 0.05</td>
<td>-</td>
</tr>
<tr>
<td>YY753/758FF</td>
<td>145 $\pm$ 4.3</td>
<td>-21.89 $\pm$ 0.07</td>
<td>-</td>
</tr>
<tr>
<td>YY753/758FF pY778</td>
<td>1,217 $\pm$ 62</td>
<td>-16.63 $\pm$ 0.13</td>
<td>5.26</td>
</tr>
</tbody>
</table>

$^a$ $K_d$ values are given ± standard error.
$^b$ $\Delta G$ is given for binding and calculated from $K_d$.
$^c$ $\Delta \Delta G_{pY}$ is the $\Delta G$ value for the phosphorylated integrin binding to talin1, minus the $\Delta G$ value for the unphosphorylated integrin binding to talin1 (a positive value denotes a decrease in affinity upon phosphorylation).
$^d$ Approximate $K_d$ values were estimated by comparing the magnitude of chemical shift perturbations to those in a relevant titration, as described in Experimental Procedures.
Figure 3.4: Effect of tyrosine phosphorylation on the integrin/talin1 interaction.

Weighted chemical shift maps of perturbations observed in $^1$H-$^{15}$N HSQC spectra of the (A) β3, (B) β1A, and (C) β7 tails (50 μM) upon the addition of talin1 F3 domain (1 mM). Interaction studies were performed on unphosphorylated integrin tails and tails phosphorylated at the nMD site (β3, β1A, and β7) and at the fMD site (β3). Grey bars correspond to residues that could not be tracked due to exchange broadening. Note that the y-axis scale differs between panels.
A

Δδ(H,N,N) (ppm)

β3

Tyr(P)747

Tyr(P)759

716 726 736 746 756

B

Δδ(H,N,N) (ppm)

β1A

Tyr(P)783

752 762 772 782 792

C

Δδ(H,N,N) (ppm)

β7

Tyr(P)778

747 757 767 777 787 797

β tail residue
Tyrosine phosphorylation increases integrin affinity for Dok1.

Unlike the talin1 F3 domain, the PTB domain of Dok1 only caused small perturbations in the HSQC spectra of unphosphorylated integrin tails. Such perturbations were localized to the nMD region of β3 and the fMD regions of β1A and β7. However, these interactions were so weak (K_d greater than several mM) that it is unlikely that any of these interactions are physiologically relevant (Table 3.2). It is of note that although the K_d value for β3 (12.6 mM) is only an estimate, as described in Experimental Procedures, it agrees well with the 14.3 mM value reported for a short fragment of β3 (Oxley et al., 2008).

Table 3.2: Effect of tyrosine phosphorylation on the affinity of the integrin/Dok1 interaction

<table>
<thead>
<tr>
<th>Mutation</th>
<th>K_d (μM)^a</th>
<th>ΔG (kJ/mol)^b</th>
<th>ΔΔG_pY (kJ/mol)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>β3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>12,600 est.</td>
<td>-10.8</td>
<td>-</td>
</tr>
<tr>
<td>Y759F pY747</td>
<td>20.9 ± 2.9</td>
<td>-26.69 ± 0.34</td>
<td>-15.9</td>
</tr>
<tr>
<td>Y747F pY759</td>
<td>226 ± 7.7</td>
<td>-20.80 ± 0.08</td>
<td>-10.0</td>
</tr>
<tr>
<td>β1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>N/A</td>
<td>-23.41 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>pY783</td>
<td>78.7 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>N/A</td>
<td>-25.30 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>YY753/758FF</td>
<td>36.8 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pY778</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a K_d values are given ± standard error.
^b ΔG is given for binding and calculated from K_d.
^c ΔΔG_pY is the ΔG value for the phosphorylated integrin binding to Dok1, minus the ΔG value for the unphosphorylated integrin binding to Dok1 (a negative value denotes an increase in affinity upon phosphorylation)
Upon phosphorylation, the affinities of these interactions increased substantially. For phosphorylation in the nMD regions, $K_d$ values ranged from 20.9 μM for β3 to 36.8 μM for β7 to 78.7 μM for β1A (Table 3.2). Phosphorylation of β3 Y759 also increased Dok1 affinity to a $K_d$ of 226 μM. In each case, the interaction as observed by NMR was localized to residues near the site of phosphorylation. No MP perturbations were observed in β3 or β1A, and only minor MP perturbations in β7 (Figure 3.5). Tyrosine phosphorylation thus greatly increases the affinity of Dok1 for integrin tails (adding 16 kJ/mol of binding energy to the interaction in the case of β3 pY747), making the interaction tight enough to be physiologically relevant and significantly tighter than the competing talin/integrin interaction.
Figure 3.5: Effect of tyrosine phosphorylation on the integrin/Dok1 interaction.

Weighted chemical shift maps of perturbations observed in $^1$H-$^{15}$N HSQC spectra of the (A) $\beta_3$, (B) $\beta_1A$, and (C) $\beta_7$ tails (50 $\mu$M) upon the addition of Dok1 PTB domain (1 mM). Interaction studies were performed on unphosphorylated integrin tails and tails phosphorylated at the nMD site ($\beta_3$, $\beta_1A$, and $\beta_7$) and at the fMD site ($\beta_3$).
Positively-charged residues in the NPxY binding pocket make Dok1 specific for phosphorylated integrin tails.

When we solved the crystal structure of the human Dok1 PTB domain (PDB 2V76) (Oxley et al., 2008), we found a sulfate anion in the NPxY binding pocket, surrounded by a collection of positively-charged residues: R207, R208, R222, and R223; R207 and R222 make the most direct contact (Figure 3.2A). We hypothesized that these positively-charged residues may explain the higher affinity of Dok1 for phosphorylated β3; we tested this hypothesis by individually mutating these residues to alanine. These mutations only had minor effects on the affinity of Dok1 for unphosphorylated β3 tail, but the effect was much more pronounced on the interaction with β3 tail phosphorylated at Y747 (Table 3.3). R207A had the greatest effect (increasing the $K_d$ of the interaction with phosphorylated β3 from 20.9 μM to 1,485 μM), followed by R222A (increasing the $K_d$ to 398 μM).

The degree to which each residue contributes to Dok1 phosphotyrosine specificity was calculated by subtracting the change in $\Delta G$ caused by the R-to-A mutation for the interaction with unphosphorylated tail from the change in $\Delta G$ for the interaction with phosphorylated tail (yielding the value $\Delta \Delta G_{RA/pY}$ in Table 3.3); the additive loss of phosphotyrosine-specific affinity for all four mutations (20.8 kJ/mol) is more than enough to explain the 15.9 kJ/mol increase in binding affinity of Dok1 upon β3 phosphorylation at Y747. R207 and R222 are the greatest contributors to phosphotyrosine specificity (44% and 30%, respectively), but R223 and R208 also play a role (17% and 9%, respectively).
## Table 3.3: Disrupting Dok1 binding to phosphorylated β integrin tails

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$K_d$ (μM)$^a$</th>
<th>$\Delta G$ (kJ/mol)$^b$</th>
<th>$\Delta\Delta G_{RA}$ (kJ/mol)$^c$</th>
<th>$\Delta\Delta G_{PY}$ (kJ/mol)$^d$</th>
<th>$\Delta\Delta G_{RA/PY}$ (kJ/mol)$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β3 WT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dok1 WT</td>
<td>12,600</td>
<td>est.$^g$</td>
<td>-10.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dok1 R207A</td>
<td>22,800</td>
<td>est. -9.4</td>
<td>1.5</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Dok1 R208A</td>
<td>22,600</td>
<td>est. -9.4</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dok1 R222A</td>
<td>18,600</td>
<td>est. -9.9</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dok1 R223A</td>
<td>13,900</td>
<td>est. -10.6</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>β3 Y759F pY747</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dok1 WT</td>
<td>20.9 ± 2.87</td>
<td>-26.69 ± 0.34</td>
<td>-15.9 -6.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Dok1 R207A</td>
<td>1,485 ± 64</td>
<td>-16.14 ± 0.11</td>
<td>10.56 -6.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Dok1 R208A</td>
<td>77.3 ± 1.3</td>
<td>-23.46 ± 0.04</td>
<td>3.24 -14.1</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Dok1 R222A</td>
<td>398 ± 18</td>
<td>-19.40 ± 0.11</td>
<td>7.30 -9.5</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Dok1 R223A</td>
<td>101 ± 3.3</td>
<td>-22.80 ± 0.08</td>
<td>3.89 -12.2</td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $K_d$ values are given ± standard error.
$^b$ $\Delta G$ is given for binding and calculated from $K_d$.
$^c$ $\Delta\Delta G_{RA}$ (kJ/mol) is the $\Delta G$ value for the integrin binding to mutant Dok1, minus the $\Delta G$ value for binding to Dok1 WT (a positive value denotes a decrease in affinity upon mutation).
$^d$ $\Delta\Delta G_{PY}$ (kJ/mol) is the $\Delta G$ value for the phosphorylated integrin binding to Dok1, minus the $\Delta G$ value for the unphosphorylated integrin binding to Dok1 (a negative value denotes an increase in affinity upon phosphorylation).
$^e$ $\Delta\Delta G_{RA/Py}$ (kJ/mol) is the $\Delta\Delta G_{PY}$ value for the integrin binding to mutant Dok1, minus the $\Delta\Delta G_{PY}$ value for binding to Dok1 WT.
$^f$ $\Delta\Delta G_{mut}$ (%) is the percentage of phosphorylated integrin-specific binding energy lost by the given mutation.
$^g$ Approximate $K_d$ values were estimated by comparing magnitude of chemical shift perturbations to those in a relevant titration, as described in Experimental Procedures.

Residues R207 and R208 correspond to positively charged residues in talin, but R222 and R223 do not. Residue R223 corresponds to Y373 in talin1 and Y376 in talin2—a residue that plays a significant role in binding integrins, particularly β1 integrins (Anthis $et$ $al.$, in preparation). Interestingly, however, R222 corresponds to a negatively charged residue (D372 in talin1, E375 in talin2, Figure 3.2). Thus, R222 was chosen as a particularly suitable candidate for
exploring differences in specificity for phosphorylated integrin tails between Dok1 and talin.

**Engineering talin to bind preferentially to phosphorylated integrin tails.**

As discussed above, Dok1 R222 has a reversed charge in talin (D372 in talin1, E375 in talin2) and is a key residue for determining Dok1 specificity for phosphorylated integrins. Consistent with this, introduction of the mutation D372R in talin1 substantially increased its binding affinity for integrin tails phosphorylated at the nMD site: 33.1 μM for β3 pY747 and 128 μM for β1A pY783 (Table 3.4).

### Table 3.4: Engineering talin1 to preferentially bind to phosphorylated integrin tails

<table>
<thead>
<tr>
<th>Mutation</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔG (kJ/mol)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔΔG&lt;sub&gt;DR&lt;/sub&gt; (kJ/mol)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ΔΔG&lt;sub&gt;pY&lt;/sub&gt; (kJ/mol)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β3 + talin1 F3 D372R</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>86 ± 3.3</td>
<td>-23.18 ± 0.09</td>
<td>-2.85</td>
<td>-</td>
</tr>
<tr>
<td>Y759F pY747</td>
<td>33.1 ± 1.6</td>
<td>-25.56 ± 0.12</td>
<td>-8.52</td>
<td>-2.38</td>
</tr>
<tr>
<td><strong>β1A + talin1 F3 D372R</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>793 ± 24</td>
<td>-17.69 ± 0.07</td>
<td>1.19</td>
<td>-</td>
</tr>
<tr>
<td>pY783</td>
<td>128 ± 5.1</td>
<td>-22.21 ± 0.10</td>
<td>-7.4</td>
<td>-4.52</td>
</tr>
</tbody>
</table>

<sup>a</sup> K<sub>d</sub> values are given ± standard error.
<sup>b</sup> ΔG is given for binding and calculated from K<sub>d</sub>.
<sup>c</sup> ΔΔG<sub>DR</sub> is the ΔG value for integrin binding to talin1 D372R, minus the ΔG value for binding to talin1 WT (a negative value denotes an increase in affinity upon mutation).
<sup>d</sup> ΔΔG<sub>pY</sub> is the ΔG value for the phosphorylated integrin binding to talin1, minus the ΔG value for the unphosphorylated integrin binding to talin1 (a negative value denotes an increase in affinity upon phosphorylation).
Interestingly, this mutation also affected talin1 binding to unphosphorylated integrin tails in different ways, although to a lesser degree. Talin1 D372R binds more tightly to unphosphorylated β3 than does talin1 WT (86 μM vs. 273 μM), but binds more weakly to unphosphorylated β1A than talin1 WT (793 μM vs. 491 μM). These differences can be explained based on recent structural data (Figure 3.2) and could influence the biological activity of this mutant.

**Talin D372R localizes to phosphorylated integrins in live cells**

Due to its dramatic effect on talin binding to phosphorylated integrins, we hypothesized that the D372R mutation would affect talin activity *in vivo*; we investigated this by examining talin localization to focal adhesions. We transiently expressed GFP-talin1 WT or D372R in SYF MEFs (deficient for the tyrosine kinases Src, Yes, and Fyn), which were then plated on fibronectin (an extracellular ligand for α5β1) and stained for the focal adhesion marker vinculin. Talin1 D372R was abundantly expressed (Figure 3.6D) but was not seen at the sites of focal adhesions, whereas talin1 WT co-localized with vinculin and was therefore present in focal adhesions (Figure 3.6A). Focal adhesions were also more prominent in cells expressing talin1 WT compared to those expressing talin1 D372R.

In SYF MEFs stably reconstituted with c-Src (SYF + Src), talin1 WT was also targeted to focal adhesions, but these adhesions were less prominent than in cases where c-Src was absent (Figure 3.6B). However, talin1 D372R in SYF +
Src MEFs was targeted to focal adhesions, and these were more prominent than those observed in SYF MEFs expressing talin1 D372R or SYF + Src MEFs expressing talin1 WT. To confirm the levels of phosphorylation in SYF MEFs and SYF + Src MEFs, fixed cells were stained with an anti-phosphotyrosine antibody (pY100), and an anti-paxillin antibody to mark focal adhesions (Figure 3.6C). Greater phosphorylation was observed in the focal adhesions of SYF + Src MEFs when compared to SYF MEFs. In agreement with our structural model, these data suggest that talin1 D372R is capable of competing with endogenous talin for integrin binding only when integrins are tyrosine phosphorylated. Furthermore, tyrosine phosphorylation appears to affect cell morphology in a manner that is reversed by the D372R mutation.
Figure 3.6: Talin D372R preferentially localizes to focal adhesions that are tyrosine phosphorylated.

(A) SYF MEFs and (B) SYF + Src MEFs transiently expressing GFP-talin1 wild type (WT) or D372R were allowed to adhere to fibronectin-coated coverslips and stained to visualize vinculin. Depicted are the localization of talin (green) and vinculin (red). (C) SYF MEFs and SYF + Src MEFs were stained to visualize phosphotyrosine (pY100, red) and paxillin (green). (D) SYF cells expressing GFP-talin1 and GFP-talin1 D372R were lysed and analyzed by Western blotting to confirm comparable expression of D372R and WT talin1.
DISCUSSION

Here, we have demonstrated that key interactions involving the β3, β1A, and β7 integrin cytoplasmic tails are similarly affected by tyrosine phosphorylation. Phosphorylation at the nMD site in each tail decreases its affinity for talin; in contrast, phosphorylation greatly increases the affinity for Dok1 (by 15.9 kJ/mol in the case of β3). The interaction of Dok1 is localized to the NPxY region of the integrin tail, even when the affinity is relatively high (20.9 μM for β3 pY747). Talin, on the other hand, also binds to the MP region, a unique interaction that is essential for integrin activation (Wegener et al., 2007). Thus, tyrosine phosphorylation acts to decrease integrin activation both by decreasing talin affinity and by increasing the affinity of competing proteins incapable of activating the integrin. This is consistent with our recent reports on β3 (Oxley et al., 2008) and an early report on β1 (Tapley et al., 1989), although our current report is the first detailed analysis of tyrosine phosphorylation across different integrins using structural biological methods.

By performing these studies on intact full-length integrin tails, we have been able to show definitively where these interactions are localized within the cytoplasmic tails, and we can look at the effect of independently phosphorylating different tyrosine residues within the same peptide. In β3, we could phosphorylate either Y747 or Y759; in either case, the interaction with Dok1 was localized only to the site of phosphorylation. Whereas phosphorylation at Y747 disrupted the interaction with talin, phosphorylation at Y759 did not. Thus, the
role of phosphorylation at this fMD site is probably not related to integrin activation; the integrin tail is very flexible in this region (Ulmer et al., 2001), and binding of Dok1 here would not be expected to compete with talin binding. The effect of phosphorylation on Dok1 binding was an order of magnitude higher at the Y747 site in β3, also demonstrating that the nMD site may play a more central role in Dok1 signaling.

This is the first publication of the talin-induced chemical shift perturbation map for β7, a lymphocyte-specific integrin (Shaw and Brenner, 1995), expanding our studies performed on β3 and β1A (Anthis et al., 2009). While showing some subtle differences to the interaction of talin with β3 and β1A, the interaction with β7 is broadly similar in that it consists of both MP and nMD interactions. In fact, the chemical shift perturbation map appears intermediate between those of β3 and β1A. In studying talin binding, it was found to be particularly important to use full-length peptides, given the large interaction surface. For example, in our previous study we reported the \( K_d \) of the interaction between talin and a short β3 peptide to be 3.49 mM (unphosphorylated) and 6.53 mM (phosphorylated) (Oxley et al., 2008), while the values for the full-length β3 peptide reported here are, respectively, 0.273 mM and 1.03 mM. With respect to Dok1, which engages a more limited interaction surface, the values are more comparable between truncated and full-length tails.

These experiments could only be carried out on phosphorylated integrin tails, as a suitable phosphomimetic mutation does not exist for studying tyrosine phosphorylation. In fact, we show here that mutating Y747 in β3 to glutamate has
the same effect as mutating that residue to alanine, which effectively abrogates protein-protein interactions in that region. This is observed for interactions with Dok1 and talin1, but in the case of Dok1 phosphorylation of Y747 strongly enhances binding—demonstrating that in this system at least, glutamate does not mimic phosphotyrosine. Any use of glutamate or aspartate as a phosphotyrosine mimic should therefore be extensively validated—at the very least by comparing it to the effect of an alanine substitution. The results of a rudimentary search through the recent literature suggest that this is not common practice, and recent studies that did compare a “phosphomimetic” Y-to-E (or Y-to-D) mutant with a Y-to-A mutant found that the two different mutations had the same effect on the system under study (Potter et al., 2005), meaning that the observed effects cannot necessarily be interpreted as being phosphorylation specific. This should not be a surprising result, given the lack of structural similarity between glutamate and phosphotyrosine (Figure 3.1B).

Here, we show that mutation of positively-charged residues in the NPxY-binding pocket of Dok1 (Figure 3.2A) (Oxley et al., 2008) disrupts Dok1 binding to the β3 integrin tail in a phosphorylation-specific manner. It has been reported that the mutation of two of these residues to alanine (RR207/208AA) decreases Dok1 binding to β3 and disrupts Dok1 signaling (Ling et al., 2005). We can now explain this observation in terms of interference with the positively-charged pocket necessary for specific binding of phosphorylated integrins—particularly R207, which explains 44% of Dok1 phosphotyrosine specificity (Table 3.3). The analogous residues in talin1 and talin2, however, are also positively charged, so
this does not explain why Dok1 binds specifically to phosphorylated integrins while talin does not. Another residue in the Dok1 NPxY-binding pocket, R222, is oppositely charged in talin (D372 in talin1, E375 in talin2). Mutation of this residue in Dok1 to alanine significantly decreases the affinity of the interaction with the β3 integrin in a phosphorylation dependent manner, and is the largest contributor to Dok1 phosphotyrosine specificity after R207.

We hypothesized that this understanding of the structural basis of the phosphorylation dependence of Dok1 could be used to engineer a talin variant that would bind specifically to phosphorylated integrins. Indeed, this effect was observed for talin1 D372R. This mutation increased talin affinity for phosphorylated β3 by 8.5 kJ/mol and β1A by 7.4 kJ/mol. Interestingly, this mutant had differing effects on the interaction with unphosphorylated integrins: it increased talin1 affinity for unphosphorylated β3 by 2.9 kJ/mol but decreased affinity for β1A by 1.2 kJ/mol. An examination of the structures of β3 and β1D in complex with talin (Figure 3.2) explains this difference. In the β3/talin1 structure (PDB 1MK9) (Garcia-Alvarez et al., 2003), the portion of the tail C-terminal to Y747 does not make extensive contacts with talin. However, this region contains a negatively-charged glutamate residue (E749) that could make favorable electrostatic contacts with the mutant D372R residue. β1A, on the other hand, has an uncharged serine residue (S785) in this position. In the β1D/talin2 structure (PDB 3G9W) (Anthis et al., 2009), S785 forms a hydrogen bond with talin2 E375. In the similar β1A/talin1 complex this mutation would disrupt such an interaction if present and would not be expected to make the interaction more
favorable as in the case of β3. This interaction between the β1 Y783 side chain and a negatively charged talin residue may also explain why the introduction of a phosphate group here has a slightly greater disruptive effect on the β1A/talin1 interaction than on the β3/talin1 interaction (Table 3.1). In addition, the E749/S785 substitution may explain why Dok1 has a higher affinity for β3 than for β1A or β7 (which also has an uncharged residue, S780, at this position), as E749 in β3 would form favorable electrostatic interactions with the positively charged Dok1 binding pocket.

We tested aspects of our structural model by observing the behavior of talin1 WT and D372R in live cells. In MEFs that do not express Src or related kinases, talin1 WT localized to focal adhesions, but talin1 D372R did not. As these experiments were carried out using fibronectin, and α5β1 is the primary fibronectin receptor in MEFs (Hodivala-Dilke et al., 1999), this effect correlates with the decreased affinity observed between mutant talin and unphosphorylated β1A integrins. When c-Src was introduced into these cells, both talin1 WT and D372R co-localized with phosphorylated integrins at focal adhesions. Thus, the effects observed in in vitro binding experiments translate into observable effects in live cells. Interestingly, tyrosine phosphorylation correlates with reduced focal adhesion formation in the presence of talin1 WT but not talin1 D372R. We did not assess integrin inside-out activation levels directly, but the increased prominence of focal adhesions in c-Src-expressing cells in the presence of talin1 D372R compared to talin1 WT provides evidence that this talin mutant disrupts the integrin activation phosphorylation switch, causing integrins to remain active
even after integrin tyrosine phosphorylation. Thus, these results provide additional evidence that integrin tyrosine phosphorylation downregulates integrin activation by inhibiting talin binding.

Phosphorylation of the nMD tyrosine residue has been shown to modulate inside-out integrin activation in β3 (Datta et al., 2002) and in β1 (Tapley et al., 1989). In β3, phosphorylation of both nMD Y747 (Blystone et al., 1997; Law et al., 1999; Chandhoke et al., 2004; Gao et al., 2005; Butler and Blystone, 2005) and fMD Y759 (Cowan et al., 2000; Kirk et al., 2000; Xi et al., 2006) have been associated with outside-in signaling β3. This is consistent with a report that the nMD region of β3 in general is associated with both inside-out and outside-in signaling, while the fMD portion only engages in outside-in signaling (Zou et al., 2007). This makes sense from a structural standpoint, in that talin only interacts tightly with the nMD NPxY. For β1, studies point to a role for phosphorylation at nMD Y783 in signaling in both directions. Evidence for a signaling role for Y795 phosphorylation is sparser, but also exists (Sakai et al., 2001; Wennerberg et al., 2000). However, under the conditions used in this study, c-Src did not phosphorylate this residue, so a different kinase may be involved in vivo. Interestingly, in β7 this fMD residue is a non-phosphorylatable phenylalanine.

Despite the substantial evidence for a role for tyrosine phosphorylation in β1 integrin signaling, mice with the β1 YY783/795FF knock-in mutation do not display any apparent developmental abnormalities (Chen et al., 2006; Czuchra et al., 2006). The β3 YY747/759FF mouse, however, displays a severe phenotype (Law et al., 1999). β1 tyrosine phosphorylation is central to the pathological effect
of v-Src on cells (Hirst et al., 1986; Johansson et al., 1994; Sakai et al., 2001), but given that these residues are so highly conserved across different integrins and across different species, it is unlikely that such conservation would exist if the residue only participated in pathological conditions. In the case of β3, at least, mutation of either NPxY tyrosine to phenylalanine had little effect on the interaction with talin1, so it is reasonable to hypothesize that these residues are conserved as tyrosine because of a role for phosphorylation—although the side chains of unphosphorylated β1D Y783 (Anthis et al., in preparation) and β3 Y747 (Garcia-Alvarez et al., 2003) do also participate in intermolecular hydrogen bonds in complex with talin. The β1 YY783/795FF mutation affects cell behavior in tissue culture conditions (Sakai et al., 1998; Wennerberg et al., 2000), so the lack of a phenotype in β1 YY783/795FF knock-in mice may be the result of compensation by other integrins. On the other hand, the more extreme effect of tyrosine phosphorylation on Dok1 binding to β3 correlates with the more definitive biological role observed for tyrosine phosphorylation in that integrin.

Several studies have provided evidence of a biological role for a direct interaction between Dok1 and β3 integrins (Wegener et al., 2007; Ling et al., 2005; Clemmons and Maile, 2005; Senis et al., 2009), but what role Dok1 plays in the signaling of other integrins remains more of an open question. A variety of other PTB domains have been identified that also interact with integrins (Calderwood et al., 2003), and it is possible that one of these other proteins acts as a phosphorylation-dependent activation switch for β1 or other integrins. The PTB domain of ICAP-1, for example, has been identified as a phosphorylation-
independent downregulator of \( \beta_1 \) activation, but given the large number of PTB domains that bind preferentially to phosphorylated substrates (Uhlik et al., 2005), other phosphorylation-dependent down-regulators may also exist. Given this uncertainty, however, and the results of the \( \beta_1 \) YY783/795FF knock-in mouse (Chen et al., 2006; Czuchra et al., 2006), this is an area that will require further study.

From the current study, and from the bulk of structural and biological data on the topic, integrin tyrosine phosphorylation appears to be involved in a wide variety of integrin signaling processes, particularly for \( \beta_3 \), but for other integrins as well. We demonstrate here a conserved structural mechanism in \( \beta_3 \), \( \beta_1 \)A, and \( \beta_7 \) integrins for regulation of integrin activation by nMD tyrosine phosphorylation. We have tested our predictions by engineering a talin mutant that is specific for phosphorylated integrins, and we have shown that this influences talin localization in live cells. Given that the literature on integrin tyrosine phosphorylation is substantial but sometimes ambiguous, our results add weight to the idea that tyrosine phosphorylation plays a significant role in integrin signaling.

Chapter 3, in full, is a reprint of the material as it appears in The Journal of Biological Chemistry, 284(52), 36700-10 (2009). Anthis, N; Haling, JR; Oxley, CL; Memo, M; Wegener, KL; Lim, CJ; Ginsberg, MH; Campbell, ID. Jacob Haling was the secondary investigator and author of this paper.
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Chapter 4

The ERK1/2 MAP kinase pathway suppresses integrin activation by phosphorylating FRS2α.

INTRODUCTION

Integrin adhesion receptors are heterodimeric transmembrane proteins consisting of an α and β subunit that link the extra-cellular matrix (ECM) with the intra-cellular cytoskeleton and transmit signals through this connection. Thus, integrins play central roles in cell adhesion, migration, differentiation, and proliferation (Hynes, 2002). Integrin activation is here defined as an increase in integrin affinity for its cognate ligands. Signals from within the cell that activate integrins are referred to as inside-out signaling, to contrast with outside-in signaling that results from the binding of ligands that lead to intracellular signals. Integrin cytoplasmic domains (tails), in particular highly conserved NPxY motifs of β integrins, are important for the binding of proteins that regulate inside-out integrin activation (Du et al., 1993; O'Toole et al., 1994; Calderwood et al., 1999). Binding of the protein talin to the β integrin cytoplasmic domain is a final step in the activation of integrins (Tadokoro et al., 2003; Tanentzapf et al., 2006). Indeed, β integrin-talin interactions are both necessary and sufficient for integrin activation (Petrich et al., 2007; Ye et al., 2010). Recent studies have demonstrated that talin induces activation by binding first to the NPxY motif of β integrins via its phosphotyrosine binding (PTB) domain (Calderwood et al., 2002).
This docking site permits talin to then bind to a membrane proximal region of β integrins resulting in allosteric rearrangements of the αβ heterodimer causing an extracellular conformation change, and hence integrin activation (Wegener et al., 2007). Mutations in β integrins or talin that disrupt the interaction at the membrane proximal or distal sites inhibit integrin activation (Wegener et al., 2007).

Other PTB domain-containing proteins can bind to β integrin cytoplasmic tails, but in contrast to talin, they negatively regulate integrin activation (Calderwood et al., 2003). Furthermore, phosphorylation of the NPxY motif of β integrin tails can function as a molecular switch to regulate integrin activation (Ling et al., 2005; Oxley et al., 2008). For example, phosphorylation of Y747 in the β3 integrin tail causes the DOK1 PTB domain to bind much more strongly than talin does. Structural studies of DOK1 reveal that two arginine residues form a pocket for negatively charged groups such as phospho-tyrosine (Oxley et al., 2007; Anthis et al., 2009). Moreover, this arginine pocket is conserved among all IRS-1 like PTB domains. This establishes a two step mechanism where the β integrin tail is tyrosine phosphorylated, followed by the binding of a PTB containing protein(s), resulting in the loss of talin binding and the inactivation of integrins. While this represents one mode of inhibition, it is by no means the only mechanism documented to regulate integrin activation.

We previously found that HRas, Raf-1 and ERK1/2 of the MAP kinase signaling pathway inhibit integrin activation (Hughes et al., 1997). Furthermore, ERK1/2 kinase activity is required for this function, and they exert their effects at
the plasma membrane (Chou et al., 2003). Crosstalk between integrins and the ERK1/2 MAP kinase pathway is well-documented throughout the literature. ERK1/2 signaling, like integrin signaling, is implicated in cell migration, focal adhesion turnover, and matrix assembly (Klemke et al., 1997; Brenner et al., 2000; Glading et al., 2000; Huang et al., 2004). The dysfunction of which is a hallmark of a variety of diseases including atherosclerosis and cancer (Lal et al., 2009; Desgrosellier and Cheresh, 2010). Thus, elucidating the molecular mechanism of how ERK1/2 suppresses integrin activation may provide insight into how these two different signaling cascades converge and influence important biological processes.

Fibroblast growth factor (FGF) receptor substrate 2 (FRS2α) is an adaptor/scaffold protein that contains an N-terminal myristoylation site for localization to the plasma membrane and binds to a variety of receptor tyrosine kinases (RTKs) through its PTB domain (Schlessinger, 2000; Gotoh, 2008). Upon growth factor signaling FRS2α undergoes tyrosine phosphorylation, generating six functional pTyr-binding sites. These pTyr-sites function to form a complex with the tyrosine phosphatase, Shp2, and the adapter protein Grb2, which associates with the Ras GEF, SOS. The FRS2α-Grb2-SOS ternary complex then activates the ERK1/2 MAP kinase cascade in a sustained manner (Wang et al., 1996; Kouhara et al., 1997; Hadari et al., 1998; Xu et al., 1998; Ong et al., 2000). Active ERK1/2 phosphorylates FRS2α on eight threonine residues leading to decreased pTyr of FRS2α and attenuates the ERK1/2 MAP kinase pathway (Lax et al., 2002; Wu et al., 2003). Thus FRS2α and ERK1/2 function to
form a negative feedback loop to limit MAP kinase activation in response to growth factor stimulation. Preventing ERK1/2 localization to the plasma membrane interrupts the negative feedback loop and results in sustained MAP kinase activation (Haling et al., 2010). Thermodynamic studies of FRS2α reveal that disruption of a sequence C-terminal to the FRS2α-PTB domain switches its ligand-binding affinity to different substrates within the cell (Yan et al., 2002). Interestingly, this region of FRS2α contains canonical ERK1/2 phosphorylation sites (PXTP) that have been implicated in conformational changes that regulate binding partners to FRS2α (Lax et al., 2002; Zhou et al., 2009).

Here we define a mechanism whereby the ERK1/2 MAP kinase pathway inhibits integrin activation. Previous structural and biochemical studies have revealed that talin, a PTB domain containing protein, binds to the cytoplasmic tail of β integrins and is sufficient for integrin activation. Because other proteins containing PTB domains can bind to the cytoplasmic tails of β integrins and inhibit integrin activation, we examined the effects of a PTB domain containing protein that is also a membrane-tethered substrate of ERK1/2, FRS2α. ERK1/2-mediated phosphorylation of FRS2α promoted the association of FRS2α with the β3 cytoplasmic tail. Moreover, the PTB domain of FRS2α (FRS2α-PTB) suppressed talin-mediated integrin activation by competing with talin for β3. NMR and molecular modeling revealed FRS2α-PTB has two distinct β3 binding regions. An arginine pocket on FRS2α-PTB contributes to an increased affinity to tyrosine phosphorylated β3, while both the arginine pocket and a membrane proximal site play a role in the binding to β3. This information allowed us to
design a mutant β3 integrin capable of talin-mediated activation, yet insensitive to the effects of FRS2α-PTB. Finally, genetic deletion of FRS2α prevented HRas suppression of integrin activation, establishing that FRS2α is required for this function of the ERK1/2 MAP kinase pathway. Thus, activation of ERK1/2 induces phosphorylation of FRS2α, which preferentially binds to tyrosine phosphorylated β integrins and competes with talin at mutual binding sites, resulting in the inhibition of integrin activation.

MATERIALS AND METHODS

Plasmids

Human FRS2α cDNA was obtained from American Type Culture Collection (Manassas, VA) and subcloned into pCDNA3.1 via polymerase chain reaction (PCR). The FRS2α mutant T8V was generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Both FRS2α constructs in pCDNA3.1 included a C-terminal hemagglutinin (HA) tag and were verified by DNA sequencing. The GFP-tagged FRS2α PTB domain (FRS2α-PTB, amino acids 13-116) and the myristoylated FRS2α PTB domain (FRS2α-mPTB, amino acids 1-116) were created by cloning into pEGFP-N1. Additional FRS2α-PTB/mPTB mutants R63E, R78E, and K90A were generated with the QuickChange site-directed mutagenesis kit. Mouse Talin-head (TalinH, amino acids 1-433) was subcloned into pCDNA3.1 via PCR with an N-terminal HA-tag.
HA-tagged cDNA expression constructs HRasG12V and ERK1-CAAX used in this work have been characterized previously (Chou et al., 2003).

**Cell Culture**

Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection. The generation of Raf-1:ER cells was described previously (Hughes et al., 1997). Mouse embryonic fibroblast (MEF) cells carrying homozygous LoxP-flanked FRS2α alleles have been described previously (Lin et al., 2007). αβpy cells are a CHO cell line that expresses the polyoma large T antigen and constitutively active chimeric integrin (αIIbβ3β1) (Baker et al., 1997). A5 cells are a CHO cell line that stably express human wild-type αIIbβ3 and have been previously described (O'Toole et al., 1990). All cells were cultured in DMEM (Lonza Walkersville, Walkersville, MD) containing 10% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin. Transient transfections of CHO, Raf-1:ER, αβpy, and A5 cells were carried out with Lipofectamine Plus (Invitrogen, Carlsbad, CA) as described by the manufacturer. MEF cells were transfected using a nucleofection device (Lonza Walkerville, Walkersville) in combination with PBS and program A-129 (Lonza Walkersville).

**Antibodies and Reagents**

Anti-His, -GST, and -FRS2α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-tyrosine was purchased from Cell
Signaling Technology (Danvers, MA). Anti-HA monoclonal antibody was purchased from Covance Research Products (Princeton, NJ). Full-length A.v. polyclonal antibody for the detection of GFP-tagged proteins was purchased from Clontech (Mountain View, CA). Unless otherwise indicated, all antibodies for Western Blotting were used at a 1:1000 dilution. The conformation-specific monoclonal antibody PAC1 has been previously described (Shattil et al., 1985). Alexa Fluor 647 goat anti-mouse IgM was purchased from Invitrogen (Carlsbad, CA). 4-Hydroxy tamoxifen (4-OHT) was obtained from Sigma-Aldrich and used at a final concentration of 300nM for the induction of Raf-1.

**In-vitro Protein Interactions**

The recombinant human talin head expression plasmid construct was obtained from Dr. Steve Lam (Patil et al., 1999). This construct, which contains amino acids 1–465 of human talin1, was truncated at residue 433 by introduction of a stop codon, and a his6 tag was added to the C terminus to facilitate purification. The protein was expressed in *Escherichia coli* BL21-DE-pLys (which produced higher protein yields) and purified with His-binding beads according to the manufacturer’s instructions (EMD). The recombinant FRS2α-PTB expression plasmid was created by cloning in amino acids 11-140 of human FRS2α into pGEX6p2 (GE Life Sciences). The protein was expressed in ArcticExpress *Escherichia coli* BL21 (Stratagene) at 12°C overnight, and purified with GST-binding beads according to the manufacturer’s instructions (GE Healthcare). The purified proteins were dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS
buffer). Mutations within the talin head were introduced with the QuikChange Mutagenesis kit (Stratagene). Recombinant integrin cytoplasmic tail proteins containing both a His$_6$ and an in vivo biotinylation peptide at the N terminus were expressed and purified from *Escherichia coli* extracts as previously described (Arias-Salgado E.G. *et al.*, 2003). Purified integrin tail proteins were immobilized on neutravidin-agarose (Pierce) according to manufacturer’s instructions. Interaction of GST-FRS2α-PTB and His-talinH with integrin tails was conducted in a reaction buffer (20mM PIPES (pH 6.8), 50mM NaCl, 0.1% Triton X-100, 150mM Sucrose, 1mM Sodium orthovanadate, 50mM Sodium fluoride, and 40mM Sodium pyrophosphate) and incubated at 4°C overnight. After washing the beads with reaction buffer, samples were fractionated on 4–20% SDS-PAGE gel (Invitrogen). Affinity chromatography of cell lysates was performed using immobilized integrin tails as previously described (Arias-Salgado E.G. *et al.*, 2003). Bound proteins were analyzed by Western blotting or Coomassie Blue staining.

**Tyrosine Phosphorylation of β3 Integrin Cytoplasmic Tails**

The expression constructs of c-Src kinase domain and YopH were generously donated by John Kuriyan’s laboratory. The kinase domain of c-Src (Gln$^{251}$–Leu$^{533}$) in pET28 was co-expressed with *Yersinia pseudotuberculosis* YopH in pCDFDuet-1 and purified by immobilized metal affinity chromatography as previously reported (Seeliger *et al.*, 2005). Tyrosine phosphorylation was performed overnight at 30 °C with 20 μm integrin tail and 0.015 mg/ml of Src in
50 mm Tris, 20 mm MgCl₂, 10 mm MnCl₂, 1 mm ATP, 1 mm dithiothreitol, pH 7.0.

Tyrosine Phosphorylation of β3 integrin cytoplasmic tails

**Integrin Activation Assays**

Using a lentiviral cloning vector pRRLSIN.cPPT.PGK-IRES-GFP.WPRE. (Addgene, plasmid ID 12252), viruses containing αIIb or β3 gene were generated separately as described previously (Wiznerowicz and Trono, 2003). CHO or MEF cells stably expressing integrin αIIbβ3 were established by co-infection of CHO-K1/MEF cells with the αIIb and β3 viruses. The Y747F, Y759F, and E726A mutations were introduced into the β3 gene with the QuikChange Mutagenesis kit. To assess the effects of TalinH on the activation of integrins, 2µg TalinH expression construct and 0.1 µg D-tomato (a transfection marker) were cotransfected. After 24 h, the cells were trypsinized, stained with PAC1 for 30 min, washed, stained with AlexaFlour647-conjugated anti–mouse IgM, and then analyzed by FACS. Separate FACS was also performed in the presence of anti-LIBS6 as full activation control and in presence 20 µM eptifibatide as negative control. To assess the effect of FRS2α-mPTB on those cells, 2µg TalinH expression construct, 4µg of FRS2α-mPTB construct, and 0.1 µg D-tomato construct were cotransfected into the cells, and the cells were stained as described above and analyzed by FACS. Integrin activation was quantitated as an activation index (AI) defined as \((F - F_r)/(F_{LIBS6} - F_r)\) in which \(F\) is the median fluorescence intensity of PAC1 binding; \(F_r\) is the median fluorescence intensity of PAC1 binding in the presence of eptifibatide; and \(F_{LIBS6}\) is the median
fluorescence intensity in the presence of anti-LIBS6. Flow cytometric assays of A5 cells and αβpy cells were performed as described earlier (Feral et al., 2005; Han et al., 2006; Lee et al., 2009) and integrin activation was calculated as described here.

**NMR Spectroscopy**

All NMR experiments were performed on spectrometers equipped with Oxford Instruments superconducting magnets (500 MHz $^1$H operating frequency) and GE Omega or Bruker TopSpin consoles. Unless otherwise indicated, samples were prepared in NMR buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM DTT, pH 6.1) with 5% D$_2$O and Complete protease inhibitors (Roche). Experiments were performed at 25°C, and spectra were referenced in the direct dimension to DSS at 0 ppm, with indirect referencing in the $^{15}$N dimension using a $^{15}$N/$^1$H frequency ratio of 0.101329118 (Wishart et al. JBNMR 1995). Data were processed using NMRPipe (47) and spectra were visualized using the program SPARKY (www.cgl.ucsf.edu/home/sparky). The $^1$H and $^{15}$N resonance assignments of the β3 and β1A integrin tails were previously assigned and deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) under accession numbers 15552 (Oxley et al. JBC 2008) and 16159 (Anthis et al. EMBOJ 2009), respectively.

$^1$H-$^{15}$N HSQC titrations of 0.05 mM $^{15}$N-labelled integrin tail were acquired in the presence and absence of 0.05 mM unlabelled GST-tagged FRS2 PTB domain or unlabelled untagged talin1 F3 domain. Interactions were probed by
measuring NMR peak intensities of the integrin tail. The reported value $I/I_0$ is the ratio of the intensity of the peak in the presence of FRS2 or talin1 divided by the intensity in the absence of any added protein. For each plot, the value of $I/I_0$ for the peak corresponding to the most C-terminal residue in the integrin tail was normalized to 1.0. Because the interactions probed in this study exhibit intermediate exchange kinetics, a decrease in peak intensity indicates that the given residue is affected by a protein-protein interaction.

**RESULTS**

**Threonine phosphorylation of FRS2α is required for the association with β3 integrin.**

β integrin cytoplasmic tails contain NPxY motifs that are potential docking sites for proteins containing PTB domains (Calderwood et al., 2002). FRS2α is a PTB domain containing protein capable of binding to a juxtamembrane region of the FGFR and to the NPxY motif of TRKs (Yan et al., 2002). Furthermore, FRS2α is phosphorylated by ERK1/2 at multiple threonine residues adjacent to the PTB domain in response to a variety of growth factors, such as FGF, insulin, EGF, and PDGF (Gotoh 2008) (Figure 4.1A). However, no study to date has identified proteins that bind as a consequence of FRS2α threonine phosphorylation. To test conditions that may permit binding of FRS2α to the β3 integrin, we used a CHO cell line containing tamoxifen-regulated Raf-1 to activate ERK1/2 (Hughes 1997) and thereby increase threonine phosphorylation
of FRS2α. Cells were transfected with cDNA’s encoding HA-tagged wild-type FRS2α or a mutant that has eight threonine residues mutated to valine (FRS2αT8V), treated with tamoxifen, and lysed. Cell lysates were subjected to affinity chromatography by the immobilized cytoplasmic tails of either β3 or αIIb integrin and bound proteins were detected by western blotting. Threonine phosphorylated FRS2α, as indicated by reduced electrophoretic mobility, preferentially bound to β3 whereas FRS2αT8V did not (Figure 4.1B). Furthermore, neither FRS2α nor FRS2αT8V bound to αIIb, establishing the specificity of the interaction with the β cytoplasmic tail. This indicates that Thr phosphorylation of FRS2α by ERK1/2 phosphorylation sites (PXTP) is required for binding to the β3 integrin tail.
Figure 4.1: Threonine phosphorylation of FRS2α promotes association with β3 integrin.

(A) Schematic diagram of how ERK1/2 MAP kinase signaling leads to ERK1/2 mediated threonine phosphorylation of FRS2α. FRS2α associates with RTK’s in response to growth factor signaling. Consequently, this results in sustained activation of the canonical ERK1/2 MAP kinase pathway leading to phosphorylation of ERK1/2. Active/phosphorylated ERK1/2 then phosphorylates FRS2α on threonine residues, resulting in attenuation of MAP kinase signaling. 

(B) Raf-ER CHO cells were transfected with cDNA’s encoding HA-tagged FRS2α or HA-tagged FRS2αT8V. After 24h, the cells were treated with 4-OHT for 30min at 37°C or left untreated. Cell lysates were incubated with beads coated with recombinant β3 or αIIb. Bound proteins were fractionated by SDS-PAGE and FRS2α was detected by Western blotting. Loading of the recombinant integrin tails on the beads was assessed by Coomassie blue staining.
A. 

Growth Factor

RTK

Plasma Membrane

PTB

FRS2

MAPK signaling

ERK1/2

B.

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<th>FRS2</th>
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30min treatment with 4-OHT

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The PTB domain of FRS2α inhibits integrin activation.

Activation of the ERK1/2 MAP kinase pathway inhibits integrin activation (Hughes et al., 1997; Ramos et al., 2000; Chou et al., 2003) independent of mRNA transcription and protein synthesis (Hughes et al., 1997). Having observed that ERK1/2 phosphorylation stimulates the binding of FRS2α to the β3 tail, we hypothesized that FRS2α could mediate ERK1/2-induced suppression of integrin activation. As a first step to test this hypothesis we used a CHO cell line (αβpy) that stably expresses constitutively active integrins (see methods). αβpy cells were transfected with the myristoylated FRS2α PTB domain (mPTB), the FRS2α PTB domain lacking the myristoylation site (PTB), or full length FRS2α. We used a talin mutant that binds to integrins but does not activate (TalinL325R) as a control for a PTB domain protein that suppresses activation. Twenty four hr after transfection we used flow cytometry to measure the binding of an integrin activation-specific antibody, PAC1. FRS2α-mPTB transfected cells showed a reduction in integrin activation, similar to that observed in cells expressing Talin L325R (Figure 4.2A). This suggests that FRS2α-mPTB competes with wild-type talin for binding to integrin tails, similar to talin L325R or HRasG12V. Interestingly, FRS2α-PTB showed weaker suppression of integrin activation, indicating that membrane localization by myristoylation contributes to an increased effect. Full length FRS2α had very little effect on integrin activation, consistent with our observation that ERK1/2 phosphorylation is required for FRS2α to bind to integrin β3 tail.
Next, we wanted to ascertain if FRS2α-mPTB can suppress the capacity of talin head domain (TalinH) to activate integrin αIIbβ3 (A5 cells). We co-expressed increasing amounts of FRS2α-mPTB with TalinH and used flow cytometry to assay PAC1 binding. Cells transfected with FRS2α-mPTB in conjunction with TalinH exhibited a decrease in PAC1 binding as indicated by the activation index (Figure 4.2C). Co-expression of TalinH and FRS2α-PTB did not alter the expression level of TalinH (Figure 4.2D). Thus, the myristoylated PTB domain of FRS2α can suppress talin-induced integrin activation.
Figure 4.2: The PTB domain of FRS2α inhibits integrin activation.

(A) αβpy cells were transfected with cDNA’s encoding GFP-tagged mPTB (4ug), PTB (4ug), wt FRS2α (4ug), Talin L325R (4ug), HA-tagged HRasG12V (2ug), or pCDNA3.1− (6ug). After 24 h, integrin activation was assayed by PAC1 binding. Shown is the mean activation index +/- SEM of three independent experiments. 

(B) Immunoblot demonstrating that the expression levels of cDNA’s from the experiments in 2A are similar. 

(C) A5 cells were co-transfected with cDNA’s encoding HA-tagged TalinH (2ug) and indicated amounts of GFP-tagged mPTB. After 24 h, integrin activation was assayed by PAC1 binding. Shown is the mean activation index +/- SEM of three independent experiments. 

(D) Immunoblot demonstrating that expression of GFP-tagged mPTB does not reduce expression of HA-tagged TalinH from experiments in 2C.
A.

B.

C.

D.
FRS2α-PTB competes with TalinH for direct binding to β3 integrin.

We next sought to ascertain whether the FRS2α PTB domain can compete with the talin head domain for binding to the β3 cytoplasmic tail. Recombinant TalinH (40nM) and increasing amounts of recombinant FRS2α-PTB were subjected to affinity chromatography with immobilized β3 cytoplasmic tails, and bound proteins were resolved by SDS-PAGE and quantified by western blotting followed by scanning densitometry. 500nM of FRS2α-PTB was sufficient to reduce TalinH bound to β3 (Figure 4.3). Furthermore, gradually increasing the amount of FRS2α-PTB up to 5μM further reduced TalinH-β3 binding. Therefore, FRS2α-PTB can directly compete with TalinH for binding to recombinant β3 integrin tail.
Figure 4.3: FRS2α-PTB competes with Talin-Head for binding to β3 integrin.

Increasing amounts of recombinant GST-FRS2α PTB domains, ranging from 250nM to 5μM, were mixed with 40nM of recombinant His-TalinH and beads coated with recombinant β3 tails. Bound proteins were fractionated by SDS-PAGE and detected by Western blotting. Levels of His-TalinH bound to β3 were quantified by scanning densitometry and the values are displayed above the protein band. Note that the amount of His-TalinH bound to β3 gradually decreases with increased FRS2α-PTB loading. Loading of the recombinant integrin tails on the beads was assessed by Coomassie blue staining.
Molecular modeling and mutagenesis confirm that canonical PTB-ligand interactions contribute to the integrin/FRS2α interaction.

To gain further insight into the integrin tail/FRS2α-PTB interaction, we built a homology model of β3 tail/FRS2α-PTB complex based on the known structure of β1D/TalinF2F3 complex. This model of β3/FRS2α-PTB complex was energy minimized using NAMD, a molecular mechanics program. FRS2α-PTB was predicted to interact with β3 in a manner resembling other PTB-ligand interactions with the tyrosine of the NPxY motif (Y747) within β3 projecting into an arginine pocket of FRS2α-PTB (Figure 4.4A). To confirm this interaction, we measured the binding of recombinant FRS2α-PTB to β3Y747A. FRS2α-PTB bound only to wild type β3, indicating that Y747 is necessary for this interaction (Figure 4.4B). Thus, FRS2α binds to the β3 tail in a manner resembling a PTB-ligand interaction; the capacity of FRS2α PTB domain to compete with talin is explained by the fact that talin also binds β3 via Tyr 747 in a similar manner.

The integrin/FRS2α-PTB interaction is enhanced by integrin tyrosine phosphorylation.

The effect of β1A integrin Tyr783 phosphorylation and β3 integrin Tyr747 phosphorylation on binding to the FRS2α PTB domain was studied by nuclear magnetic resonance (NMR). For each peak the intensity of integrin tail peptide in the presence of FRS2α-PTB (I) was divided by the intensity for the integrin tail peptide alone (I0) and normalized so that I/I0 for the most C-terminal residue was equal to 1. Thus, a reduction in peak intensity indicates a protein-protein
interaction near that residue. The data suggested strong interactions of β3 residues 720 and 750 with FRS2α PTB domain. These interactions had been predicted by the previously described molecular modeling and further confirmed that the FRS2α-integrin interaction resembles a canonical PTB domin-peptide interaction. Phosphorylation of both β1A and β3 resulted in decreased peak intensity between integrin residues 773-783 and 734-754 of β1A and β3 respectively (Figure 4.5A, 4.5B). These regions surround the NPxY motif which is the site of phosphorylation. Many PTB domains exhibit a similar increased affinity for Tyr phosphorylated ligands due to the presence of basic residues in the Tyr binding pocket. To test whether the arginine pocket of FRS2α-PTB is required for interaction with tyrosine phosphorylated β3, we mutated the arginines to alanines (diRA) or glutamic acids (diRE). While FRS2α-PTBdiRA and FRS2α-PTBdiRE still bound to β3, tyrosine phosphorylation of the β3 cytoplasmic tail no longer increased binding as it did with wild-type FRS2α-PTB (Figure 4.4C). Thus, the arginine pocket of FRS2α-PTB is necessary for enhanced binding to phosphorylated integrin tails. Furthermore, mutation of Y747 to alanine in β3 disrupted the interaction between FRS2α-PTB and β3 (Figure 4.5C). Whereas mutations of β3 D723 or Y747 to alanine also disrupt the interaction with the talin head domain (Figure 4.5D), D723A slightly enhances binding with FRS2α-PTB, revealing a key difference between FRS2α and talin1 binding to integrins. Since D723 is an important residue for membrane proximal talin-β3 interaction, this difference explains why talin1 activates integrins while FRS2α does not.
Figure 4.4: The arginine pocket of FRS2α interacts with the NPxY motif of β3 integrin.

(A) Homology model of β3 tail (red) and FRS2 PTB domain (blue) complex was built using β1D-Talin F2F3 structure as the reference in SWISS-MODEL program server. The β3-FRS2 PTB homology model was then immersed in a sphere of water molecules and the hydrated system was energy minimized in NAMD with CHARMM force field to reach the local energy minimum. The side chains of selected residues involved in the interaction are drawn as ball-and-stick models with E726 and Y747 of β3 in orange, and K90, R63, and R78 of FRS2α-PTB in blue. (B) Recombinant GST-FRS2α-PTB was incubated with beads coated with recombinant β3, β3Y747A, and αIIb. Bound FRS2α-PTB was fractionated by SDS-PAGE and detected by Western blotting with an anti-GST antibody. Loading of the recombinant integrin tails on the beads was assessed by Coomassie blue staining. (C) CHO cells were transfected with cDNA’s encoding GFP-tagged wt FRS2α-PTB, FRS2α-PTBdiRA, or FRS2α-PTBdiRE. After 24 h, the cells were lysed and incubated with beads coated with recombinant β3 or tyrosine phosphorylated β3 (pY-β3). Bound proteins were fractionated by SDS-PAGE and FRS2α-PTB and pY-β3 were detected by Western blotting. Loading of the recombinant integrin tails on the beads was assessed by Coomassie blue staining.
A. 

B. 

C.
Figure 4.5: NMR-based studies reveal that the integrin/FRS2α interaction is enhanced by integrin tyrosine phosphorylation.

$^1$H-$^{15}$N-HSQC spectra were acquired of 50 μM $^{15}$N-labelled β integrin tail, with and without 50 μM unlabelled FRS2α or talin1 PTB domain. For each peak, the intensity in the presence of FRS2α or talin1 (I) divided was by the intensity for the integrin alone ($I_0$) and normalized so that $I/I_0$ for the most C-terminal residue was equal to 1. Values are plotted as $I/I_0$ vs. residue number, and error bars represent estimated error due to spectral noise. Because these interactions exhibit intermediate exchange kinetics, a decrease in peak intensity indicates a protein-protein interaction near that residue. (A) β1A integrin tail—unphosphorylated or phosphorylated at Y783 (pY783)—with FRS2α PTB domain. (B) β3 integrin tail—unphosphorylated or phosphorylated at Y747 (pY747)—with FRS2α PTB domain. (C) β3 integrin tail—wild type (WT), D723A, or Y747A—with FRS2α PTB domain. (D) β3 integrin tail—wild type (WT), D723A, or Y747A—with talin1 PTB domain. (E) Sequence of the cytoplasmic tail constructs of the β1A and β3 integrins used in this study. The two β3 residues mutated in panels C and D are indicated.
E  

\[ \begin{align*} 
\beta_1A & \quad 716 \quad KLLITIHDRK\ EFAKFEEERK \ RAKWD\text{ANN}P\ LYKESAVTTVV\ NPK\text{E}\text{G}K \quad 762 \\
\beta_3 & \quad 752 \quad KLLMIHDRR\ EFAKFEKEM\ NAKWD\text{GEN}P\ IYKSAVTTVV\ NPK\text{E}\text{G}K \quad 798 
\end{align*} \]
The FRS2α-PTB interaction with integrins is dependent on a membrane proximal lysine.

While mutation of the FRS2α-PTB arginine pocket did not abrogate binding to β3, NMR and molecular modeling revealed a membrane proximal interaction between FRS2α and β3 that may also participate in their association. In particular K90 of FRS2α and E726 of β3. To test this idea we introduced a K90A mutation into FRS2α-PTBdiRE (FRS2α-PTBdiREKA). CHO cells were transfected with FRS2α-PTBdiREKA, lysed, and mixed with either recombinant β3 or αIIb. FRS2α-PTBdiREKA showed a significant decrease in binding to β3, whereas FRS2αPTB-diRE and wild-type FRS2αPTB bound as expected (Figure 4.6A). We then investigated whether FRS2α-mPTBdiREKA still inhibited integrin activation. A5 cells co-transfected with TalinH and FRS2α-mPTBREKA showed only a moderate decrease of PAC1 binding compared to cells co-transfected with TalinH and wild-type FRS2α-mPTB (Figure 4.6B). Again, co-expression of TalinH did not effect the protein expression of either FRS2α-mPTB construct (Figure 4.6C). The result was more striking in αβpy cells, where FRS2α-mPTB caused a large reduction in PAC1 binding while FRS2α-mPTBREKA transfected cells had similar levels of integrin activation to cells transfected with control pCDNA3.1- alone (Figure 4.6D). Thus, K90 of FRS2α is important for binding to β integrins, and cooperates with the arginine pocket to inhibit integrin activation. Mutating these sites disrupts binding of FRS2α to β integrin tails, and hence FRS2α no longer inhibits integrin activation.
Figure 4.6: The FRS2α-PTB interaction with integrins is dependent on a membrane proximal lysine.

(A) CHO cells were transfected with cDNA’s encoding GFP-tagged wt FRS2α-PTB, FRS2α-PTBdiRE, or FRS2α-PTBdiREKA. After 24 h, the cells were lysed and incubated with beads coated with recombinant β3 or αIIb. Bound proteins were fractionated by SDS-PAGE and FRS2α-PTB was detected by Western blotting. Loading of the recombinant integrin tails on the beads was assessed by Coomassie blue staining. (B) A5 cells were co-transfected with cDNA’s encoding HA-tagged TalinH (2ug) and either GFP-tagged wt FRS2α-mPTB (4ug) or FRS2α-mPTBdiREKA (4ug). After 24 h, integrin activation was assayed by PAC1 binding. Shown is the mean activation index. (C) Immunoblot demonstrating that expression of GFP-tagged mPTB does not reduce expression of HA-tagged TalinH from experiments in 6B. (D) αβpy cells were transfected with cDNA’s encoding FRS2α-mPTBdiREKA (4ug), FRS2α-mPTB (4ug), ERK1-CAAX (4ug), or td Tomato (0.1ug). After 24 h, integrin activation was assayed by PAC1 binding. Shown is the specific binding.
A. Integrin Activation in A5 cells

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IB: GFP (FRS2)

Coomassie

B. Integrin Activation in A5 cells

![Bar graph showing integrin activation levels](image)

C. Specific Binding

IB: GFP (FRS2)

IB: HA TalinH

D. Integrin Activation in αβpy cells

![Bar graph showing integrin activation levels](image)
Selective mutations of β3 tyrosine residues prevent FRS2α-mediated suppression of integrin activation.

In order to ascertain whether the previously defined interactions involving β3 mutants and FRS2α are relevant to suppression of integrin activation, we created lentivirus constructs containing Y747F/Y759F (diYF) or Y747F/Y759F/E726A (diYFEA) β3 integrin mutants. CHO cells were transduced with αIIb and either wild-type β3, β3diYF, or β3diYFEA, followed by a transfection with TalinH and FRS2α-mPTB individually or in combination. Integrin activation was then assayed by quantifying PAC1 binding via flow cytometry. Suppression of integrin activation by FRS2α-mPTB was not observed in cells containing β3diYF, in contrast to the marked suppression of wild-type αIIbβ3 (Figure 4.7A). Furthermore, cells containing β3diYFEA had a defect in TalinH mediated integrin activation and therefore were not significantly inhibited by the co-expression of FRS2α-mPTB (Figure 4.7A). Importantly, the expression levels of TalinH and FRS2α-mPTB were comparable in αIIbβ3, αIIbβ3diYF, and αIIbβ3diYFEA expressing cells (Figure 4.7B). Thus the β3diYF mutations abrogate FRS2α mediated suppression of integrin activation.
A. Integrin Activation in β3 mutants

Figure 4.7: Tyrosine residues in the NPxY motifs of β3 integrin are required for FRS2α-mPTB inhibition of integrin activation.

(A) CHO cells were transduced with a lentivirus containing wt αIIb and either wt β3, β3 Y747F/Y759F (diYF), or β3 Y747F/Y759F/E726A (diYFEA). These cells were then transfected with cDNA’s encoding HA-tagged TalinH (2ug) and GFP-tagged FRS2α-mPTB (4ug) alone or together in indicated samples. After 24h, integrin activation was assayed by PAC1 binding. Shown is the mean activation index +/- SEM of three independent experiments. (B) Immunoblot demonstrating that expression of GFP-tagged FRS2α-mPTB does not reduce expression of HA-tagged TalinH from experiments in 7A.
FRS2α is required for HRas to suppress integrin activation.

The previous studies established that FRS2α can bind to β integrins and inhibit integrin activation. We next asked whether the capacity of the ERK1/2 MAP kinase pathway to inhibit integrin activation was dependent on FRS2α. First, we used immortalized MEFs derived from Frs2αlox/lox mice and infected them with lentivirus encoding αIIb and β3 integrin. Next, we infected the αIIbβ3 MEFs with an adenovirus encoding Cre-recombinase, resulting in the deletion of FRS2α alleles (Frs2α-/- cells). As a control, cells were infected with adenovirus encoding LacZ. The two resulting MEF cell lines were transfected with TalinH to activate integrins and with HRasG12V to induce ERK-mediated integrin suppression. In contrast to control MEFs, FRS2α-deficient MEFs did not exhibit suppression of integrin activation (Figure 4.8A). HRasG12V did not reduce TalinH expression (Figure 4.8B), and endogenous levels of FRS2α were reduced in response to Cre-recombinase (Figure 4.8C). Thus, FRS2α is required for HRasG12V to suppress talin-mediated integrin activation.
Figure 4.8: FRS2α is required for HRas to suppress integrin activation.

FRS2α-floxed MEFs were transduced with two lentiviral constructs encoding αIIb and β3. FRS2α-floxed MEFs expressing αIIbβ3 were then transduced with an adenovirus encoding Cre-recombinase or LacZ. After 48 h, cells were transfected with cDNAs encoding HA-tagged TalinH and/or HA-tagged HRasG12V, or empty vector pCDNA 3.1-. (A) 24 h post transfection, integrin activation was assayed by PAC1 binding. Shown is the mean activation index +/- SEM of three independent experiments. (B) Expression levels of HA-tagged HRasG12V and HA-tagged TalinH of samples in 8A were assayed by immunoblotting. (C) Endogenous levels of FRS2α in MEFs treated with either Cre-recombinase or LacZ were detected by Western blotting.
A. Integrin Activation in FRS2 floxed MEF’s

![Graph showing Integrin Activation in FRS2 floxed MEF’s](image)

B. Cre treated MEF’s vs. wt MEF’s

![Western Blot showing TalinH and HRasG12V expression](image)

C. Cre vs. wt

![Western Blot showing FRS2 expression](image)
DISCUSSION

The ERK1/2 MAP kinase pathway is a key component that regulates inside-out integrin activation. Activated H-Ras and its downstream effectors Raf-1 and ERK1/2 suppress integrin activation, which in-turn affects cell migration and the assembly of the extracellular matrix (Hughes et al., 1997; Huang et al., 2004; Wierzbicka-Patynowski et al., 2003). The experiments in this chapter define the mechanism whereby the ERK1/2 MAP kinase pathway inhibits integrin activation. Previous structural and biochemical studies have revealed that talin, a PTB domain containing protein, binds to the cytoplasmic tail of β integrins and is sufficient for integrin activation (Tadokoro et al., 2003; Nieswandt et al., 2007; Petrich et al., 2007; Wegener et al., 2007; Ye et al., 2010). Because other proteins containing PTB domains can bind to the cytoplasmic tails of β integrins and inhibit integrin activation (Calderwood et al., 2003), we examined the effects of a PTB domain containing protein that is also a membrane-tethered substrate of ERK1/2, FRS2α. ERK1/2-mediated phosphorylation of FRS2α promoted the association of FRS2α with the β3 cytoplasmic tail. Moreover, the PTB domain of FRS2α (FRS2α-PTB) suppressed talin-mediated integrin activation by competing with talin for β3. NMR and molecular modeling revealed FRS2α-PTB has two distinct binding regions to β3. The arginine pocket of FRS2α-PTB contributes to an increased affinity to tyrosine phosphorylated β3, while both the arginine pocket and a membrane-proximal site play a role in FRS2α-β3 integrin interactions. This information allowed us to selectively design a β3 integrin
capable of talin-mediated activation, yet insensitive to FRS2α-PTB overexpression. Finally, genetic deletion of FRS2α prevented HRas suppression of integrin activation, establishing that FRS2α is required for this function of the ERK1/2 MAP kinase pathway. Thus, activation of ERK1/2 induces phosphorylation of FRS2α, which preferentially binds to tyrosine phosphorylated β integrins and competes with talin at mutual binding sites, resulting in the inhibition of integrin activation.

Threonine phosphorylation of FRS2α promotes binding to β integrin cytoplasmic tails. Previous studies have established that FRS2α is threonine phosphorylated in response to ERK1/2 activation, which forms a negative feedback loop to attenuate MAP kinase signaling (Lax et al., 2002; Wu et al., 2003; Gotoh 2008). Furthermore, interrupting this negative feedback loop by mutating the ERK1/2 binding sites on FRS2α (Zhou et al., 2009) or by preventing membrane localization of ERK1/2 (Haling et al., 2010) prolongs FRS2α tyrosine phosphorylation. The kinetics of FRS2α tyrosine phosphorylation has profound effects on cell biology. FRS2α is one of the few adaptor proteins that can induce prolonged or transient activation of ERK1/2. For example, prolonged activation of ERK1/2 is associated with differentiation of PC12 cells, while transient activation of ERK is associated with proliferation (Marshall et al., 1995). While threonine phosphorylation of FRS2α has been thoroughly demonstrated to be a ‘shut-off valve’ for MAP kinase signaling, no additional function to date has been ascribed to this conformation of FRS2α (Gotoh 2008). FRS2α binds to the juxtamembrane region of FGF receptors lacking tyrosines or asparagines (Xu et
al., 1998; Ong et al., 2000); however a conformation change of FRS2α induces a greater affinity to the NPxpY of TRKs (Dhalluin et al., 2000; Yan et al., 2002). This conformation change is dependent on the β8 region of FRS2α, which lies between the PTB domain and ERK1/2 phosphorylation sites (PXTP).

Interestingly, FRS2α is present in cells that are deficient in RTK’s that bind to FRS2α, yet remains a substrate of ERK1/2 (Lax et al., 2002; Huang et al., 2004). Thus, it is likely that threonine phosphorylation of FRS2α may serve additional functions in addition to attenuating ERK1/2 signaling. β integrin cytoplasmic tails are attractive candidates for PTB containing proteins to bind, such as FRS2α, due to a membrane distal NPxY motif. Our finding that ERK1/2 phosphorylation of FRS2α induces association with integrins, establishes the first identified binding partner of FRS2α dependent on threonine phosphorylation.

We found that the myristoylated PTB domain of FRS2α inhibits integrin activation and ascribe this effect to the preference of FRS2α-PTB for tyrosine phosphorylated β tails and the ability of FRS2α-PTB to compete with TalinH for integrin binding. Talin is sufficient and necessary for inside-out integrin activation (Petrich et al., 2007; Ye et al., 2010) and does so by engaging the membrane-distal and membrane-proximal region of the β integrin tail (Wegener et al., 2007). This disrupts contacts between the β transmembrane helix and αIIb-transmembrane helix, separates the tails, and induces an extracellular conformational change resulting in increased binding of ligand or integrin activation (Shattil et al., 2010). Talin is unique among PTB containing proteins in that it engages both the membrane-proximal and membrane-distal regions of β
integrins (Vinogradova et al., 2002; Ulmer et al., 2003). Disruption of talin binding to either the membrane-proximal or membrane-distal region inhibits integrin activation (Wegener et al., 2007). Other PTB-domain containing proteins, such as DOK1, engage only the membrane-distal region of β integrins, and thus inhibit integrin activation (Oxley et al., 2007). Furthermore, tyrosine phosphorylation of the NPxY site on β3 integrins decreases affinity for Talin while increasing the affinity for PTB domains (Oxley et al., 2007; Anthis et al., 2009), providing a mechanism for PTB domain containing proteins to inhibit integrin activation. Our data agrees with previous work, and further demonstrates that mutation of β3 Y747 and Y759 negates FRS2α-mPTB mediated inhibition of integrin activation. The ability of FRS2α to preferentially bind to NPxpY motifs is dependent on a conformation change, as described above. However, more work is necessary to determine how threonine phosphorylation of FRS2α affects binding to known substrates. Here, we demonstrate the mechanism of how FRS2α-mPTB binds to the integrin tail, competes with talin for integrin tail binding, and thereby inhibits integrin activation.

FRS2α is similar to other adaptor proteins such as DOK, Gab, and IRS; however, the FRS2 family is unusual in that it is myristoylated and therefore constitutively membrane associated. Other membrane-linked docking proteins contain a pleckstrin homology (PH) domain, and their membrane localization is dependent on a variety of factors including the availability of specific phosphoinositides. FRS2β (FRS3) is similar in structure to FRS2α but lacks ERK1/2 phosphorylation sites (PXTP). So while FRS2α is the only ERK1/2
substrate permanently located at the plasma membrane that can inhibit integrin activation, there may be additional ERK1/2 substrates that regulate integrin function. Future studies are needed to investigate whether other membrane-linked docking proteins that contain both ERK1/2 phosphorylation sites and PTB domains (e.g. IRS2) can function similarly to FRS2α.

Our report establishes a mechanism whereby FRS2α binds to β integrins and inhibits integrin activation. As a result, this study represents the convergence of two signaling pathways that may be pertinent to a variety of biological processes. For example, focal adhesion turnover is important for cell migration to occur, and ERK1/2 has been implicated as one of the many proteins to regulate this process (Brahmbhatt et al., 2003). Our data suggests that ERK1/2 may perform this feat by phosphorylating FRS2α on threonine residues, inducing binding to β integrins and the turnover of focal adhesions. Matrix assembly is another process that has been linked to integrin function and the ERK1/2 MAP kinase pathway. The loss of fibronectin matrix correlates with the acquisition of a transformed phenotype (Hynes et al., 1992). In HT1080 cells, a Ras transformed cell line, the inhibition of ERK1/2 or the stimulation of α5β1 via an activating antibody can restore fibronectin fibril formation (Brenner et al., 2000). Perhaps threonine phosphorylated FRS2α is responsible for reduced matrix assembly by blocking integrin activation. In sum, our results establish a novel mechanism for regulating inside-out integrin activation that may be relevant to many fundamental biological processes.
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