Focal Adhesion Kinase and Paxillin Bind to Peptides Mimicking \(\beta_1\) Integrin Cytoplasmic Domains

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Abstract. The integrins have recently been implicated in signal transduction. A likely mediator of integrin signaling is focal adhesion kinase (pp125\(^{\text{FAK}}\) or FAK), a structurally distinct protein tyrosine kinase that becomes enzymatically activated upon engagement of integrins with their ligands. A second candidate signaling molecule is paxillin, a focal adhesion associated, cytoskeletal protein that coordinately becomes phosphorylated on tyrosine upon activation of pp125\(^{\text{FAK}}\). Paxillin physically complexes with two protein tyrosine kinases, pp60\(^{\text{src}}\) and Csk (COOH-terminal src kinase), and the oncoprotein p47\(^{\text{gag-crk}}\), each of which could function as part of a paxillin signaling complex. Using an in vitro assay we have established that the cytoplasmic domain of the \(\beta_1\) integrin can bind to paxillin and pp125\(^{\text{FAK}}\) from chicken embryo cell lysates. The NH\(_2\)-terminal, noncatalytic domain of pp125\(^{\text{FAK}}\) can bind directly to the cytoplasmic tail of \(\beta_1\) and recognizes integrin sequences distinct from those involved in binding to \(\alpha\)-actinin. Paxillin binding is independent of pp125\(^{\text{FAK}}\) binding despite the fact that both bind to the same region of \(\beta_1\). These results demonstrate that the cytoplasmic domain of the \(\beta\) subunits of integrins contain binding sites for both signaling molecules and structural proteins suggesting that integrins can coordinate the generation of cytoplasmic signals in addition to their role in anchoring components of the cytoskeleton.

The integrins are heterodimeric, transmembrane proteins that simultaneously bind to proteins of the extracellular matrix and to components of the actin cytoskeleton (reviewed in Albelda and Buck, 1990; Sastry and Horwitz, 1993). In addition to the obvious structural role fulfilled by these proteins, emerging evidence has implicated the integrins as active transducers of molecular signals (reviewed in Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993). Attachment of the integrins to their ligands triggers cytoplasmic changes including elevations of \([\text{Ca}^{2+}]_i\) (Richter et al., 1990; Jaconi et al., 1991; Pelletier et al., 1992; Schwartz, 1993) and pH\(_i\) (Schwartz et al., 1991a,b), the phosphorylation of cellular proteins on tyrosine (Guan et al., 1991; Kornberg et al., 1991) and the activation of at least one protein tyrosine kinase (PTK), pp125\(^{\text{FAK}}\) (Guan and Shalloway, 1992; Lipfert et al., 1992). pp125\(^{\text{FAK}}\) (focal adhesion kinase) is a unique PTK bearing a central catalytic domain flanked by large noncatalytic NH\(_2\)- and COOH-termini (Hanks et al., 1992; Schaller et al., 1992). pp125\(^{\text{FAK}}\) colocalizes with the integrins to cellular focal adhesions (Hanks et al., 1992; Schaller et al., 1992), structures that form at regions of close contact with the extracellular matrix (Burridge et al., 1988; Turner and Burridge, 1991). Amino acid sequences located near the COOH terminus of pp125\(^{\text{FAK}}\) target it to focal adhesions (Hildebrand et al., 1993). Sequences that extensively overlap with this targeting region also mediate binding to the cytoskeletal protein paxillin (Hildebrand et al., 1995). pp60\(^{\text{src}}\) and src-related PTKs physically associate with pp125\(^{\text{FAK}}\) through interactions mediated by the SH2 domains within the src-related PTKs and the major site of autophosphorylation of pp125\(^{\text{FAK}}\), tyrosine\(^{397}\) (Cobb et al., 1993; Schaller et al., 1994). The recruitment of src-like PTKs into pp125\(^{\text{FAK}}\) containing complexes may be a mechanism for amplification and/or diversification of the molecular signals that trigger the activation of pp125\(^{\text{FAK}}\).

The integrins play a central role in the formation of focal adhesions. Based upon in vitro binding data, they appear to provide anchorage for cytoskeletal proteins that in turn bind to actin filaments. Current models propose that the integrins are linked to actin via two sets of protein-protein interactions. Integrins bind to \(\alpha\)-actinin (Otey et al., 1990), which binds directly to actin (Bennett et al., 1984). Integrins also bind to talin (Horwitz et al., 1986;
Tapley et al., 1989), which binds to vinculin (Burridge and Mangate, 1984). Vinculin in turn, complexes with α-actinin (Belkin and Kotelsky, 1987; Wachstrock et al., 1987) and tensin, each of which exhibits actin-binding activity (Bennett et al., 1984; Lo et al., 1994). Thus, several focal adhesion-associated proteins have been proposed to function, at least in part, in tethering actin to the integrin at sites of focal adhesion formation. Several other proteins localize to focal adhesions including paxillin, a vinculin binding protein (Turner et al., 1990), and zyxin, an α-actinin binding protein (Crawford et al., 1992). In contrast to zyxin whose function is presently obscure, paxillin exhibits a number of interesting properties that suggests it might function in signaling. It becomes phosphorylated on tyrosine when fibroblasts spread on ECM proteins (Burridge et al., 1992) and in cells transformed by pp60v-src (Glenney and Zokas, 1989; Schaller and Parsons, 1995; unpublished observations). Furthermore, paxillin can bind to several proteins involved in oncogenic transformation and/or cytoplasmic signaling including pp60v-src (Weng et al., 1993), Csk (COOH-terminal src kinase; a PTK that phosphorylates and negatively regulates the activity of pp60v-src) (Sebe et al., 1994) and the adaptor protein p74ck (Birge et al., 1993). Csk and p74ck bind to tyrosine-phosphorylated motifs on paxillin via their src-homology 2 (SH2) domains, whereas pp60v-src binds through its src-homology 3 (SH3) domain. Thus paxillin exhibits a number of features suggesting that it functions in signaling.

The integrins themselves have no enzymatic activity and therefore must rely upon interactions with accessory proteins for the generation of cytoplasmic signals. One such candidate signaling molecule is the PTK, pp125FAK. We have considered several mechanisms by which the integrins might regulate the activity of pp125FAK and have tested the hypothesis that pp125FAK is an integrin binding protein. Using an in vitro assay we have established that the membrane proximal region of the cytoplasmic domain of the β1 integrin subunit binds directly to the NH2-terminal region of pp125FAK. The same fragment of the β1 subunit can associate with paxillin in cell lysates, although it is as yet unknown whether this interaction is direct. These data indicate that the cytoplasmic domain of the β-subunits may serve as a docking site for signaling molecules, in addition to binding to structural proteins such as talin and α-actinin, and is intimately involved in the generation of intracellular signals following stimulation with ligand.

**Materials and Methods**

**Peptide Binding Experiments**

The synthetic peptides β1 (KLMLIHierDFKEFKEKMNRAWSKTHLALRFAK), β2 (KLMLIHierDFKEFKEKMNRAWSKTHLALRFAK), and SP4 (CAVTVTVNPKYEGK) were synthesized by the Protein Chemistry Laboratory at the University of North Carolina at Chapel Hill. SP1 was purchased from Quality Controlled Biochemicals (Hopkinson, MA) as was peptide SP1 (KLMLIHierDFKEFKEKMNRAWSKTHLALRFAK). The β2 peptide (KLMLIHierDFKEFKEKMNRAWSKTHLALRFAK) was a gift of Dr. Frederick Pavalko. Mass spectrometry and amino acid analysis was carried out to confirm the sequence and purity of individual peptides. Each was coupled via its NH2-terminal cysteine residue to thiopropyl-Sepharose beads as described (Otey et al., 1990) and the extent of coupling was determined by monitoring the release of the reaction product, 2-thiopyridone (absorption coefficient = 8.08 × 105 M−1 cm−1 at 343 nm) (Stuchbury et al., 1975). After coupling, the beads were incubated with 2-mercaptoethanol to inactivate any unreacted binding sites on the beads, then extensively washed and blocked with BSA.

**Chicken embryo (CE) cells or CE cells infected with avian retroviruses containing pp125FAK residues 31-376 (nterm) or 765-1052 (cterm) were expressed in E. coli.** Expression of FAK in E. coli

Fragments of the FAK cDNA were subcloned into the vector pGex2TK (Pharmacia, Piscataway, NJ). Glutathione-S-Transferase fusion proteins containing pp125FAK residues 31-376 (nterm) or 765-1052 (cterm) were expressed in E. coli and purified using glutathione sepharose beads (Smith and Johnson, 1988). The protein kinase A site located at the junction between the glutathione-S-transferase (GST) and FAK sequencies was phosphorylated in vitro (Kaclin et al., 1992), and the FAK polypeptides were cleaved from the GST protein with thrombin (Smith and Johnson, 1988). The nterm and cterm polypeptides were then analyzed for binding as described above.

**Immunoprecipitation**

In clearing experiments, ~500 µg of lysate was incubated with either mAb 2A7 or a mAb that recognizes paxillin and the immune complexes precipitated with goat anti-mouse antibodies conjugated to agarose (Sigma). An affinity-purified rabbit antipeptide antibody (En3hance, Dupont, Boston, MA) was added to the lysate for 1 h before the addition of the β1-beads. For experiments utilizing in vitro translated polypeptides or proteins purified from Escherichia coli, the sample was diluted in modified radioimmunoprecipitation assay buffer containing 1 mg/ml BSA before addition of the beads. The beads were collected at 120 g for 2 min, washed 5-6 times with Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) and bound proteins eluted with Laemmli sample buffer (Laemmli, 1970). Eluates were analyzed by SDS-PAGE and Western blotting as described (Kanner et al., 1990) using the anti-pp125FAK mAb 2A7 (Kanner et al., 1990) or polyclonal serum BC3 (Schaller et al., 1992). Western blotting was also carried out using the anti-cortactin mAbs 1H3 or 4F11 (Kanner et al., 1990), the anti-vinulin mAb Vin 11-5 (Sigma Chem. Co., St. Louis, MO), an α-actinin antibody (a gift of Dr. Keith Burrage, University of North Carolina, Chapel Hill, NC), an anti-paxillin mAb (a gift of Dr. C. E. Turner, SUNY Health Science Center, Syracuse, NY or purchased from Transduction Laboratories, Lexington, KY) or mAb KT3 which recognizes an epitope tag on certain pp125FAK constructs (Schaller et al., 1993). Primary antibodies in Western blots were visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Radiolabeled samples were detected by autoradiography or by fluorography using Enhance (Dupont, Boston, MA).

**Results**

To test the hypothesis that pp125FAK can physically interact with the cytoplasmic domains of integrins, an in vitro binding assay was developed using a synthetic peptide, identical in sequence to the complete cytoplasmic domain of the β1 integrin subunit, coupled to thiopropyl-sepharose beads. The beads were incubated with lysates prepared from either CE cells or CE cells overexpressing pp125FAK, recovered by centrifugation and extensively washed. Proteins bound to the beads were analyzed by Western blotting (Fig. 1A). Based upon the intensities of the signals in the loading control (lane 1) and the sample bound to the beads, it can be estimated that ~10% of the pp125FAK in the lysates bound to the β1 beads. Binding was not simply due to nonspecific trapping of proteins since far less than...
10% of a second cytoskeletal protein, cortactin, bound to the β1 beads (Fig. 1 A). In fact a similar proportion of α-actinin (~10%), a known integrin binding protein, was also retained on the β1 beads (Fig. 1 A). Preincubation of the lysate with free peptide effectively inhibited binding of pp125FAK to the β1 peptide coated beads, indicating that pp125FAK bound specifically to the peptide sequence (Fig. 1 B). A number of other cytoskeletal proteins were also examined to determine the specificity of this assay. CE cell lysates (expressing only endogenous pp125FAK) were incubated with beads and binding of specific proteins was monitored by Western blotting. Vinculin exhibited a small amount of binding to β1 beads (~1-2% of the vinculin in the lysate), however, this binding was significantly less than the binding activity of α-actinin (at least 10% of the α-actinin in the lysate) (Fig. 1 C). Paxillin, in contrast, bound very efficiently to the integrin beads (Fig. 1 C). Therefore this assay detected the selective binding of several cytoskeletal proteins, including a known integrin binding protein, to a peptide modeled upon the cytoplasmic domain of the β1 integrin.

The binding of cytoskeletal proteins to the β1 peptide sequence was further characterized using four shorter peptides, each corresponding to approximately one-quarter of the full-length β1 peptide (Otey et al., 1993). Incubation of each of the peptides, coupled to beads, with cell lysates revealed that pp125FAK bound to beads coupled to short peptide 1 (SP1) but bound poorly to each of the other peptides, SP2-SP4 (Fig. 2 A). Similarly, paxillin bound efficiently only to SP1 (Fig. 2 C). pp125FAK and paxillin binding contrasts with the binding activity exhibited by α-actinin. As shown previously (Otey et al., 1993), α-actinin binds predominantly to SP2 (Fig. 2 B). The autonomously expressed COOH-terminal domain of pp125FAK, FAK-related nonkinase (FRNK), failed to bind to SP1 (Fig. 2 D). These results further underscored the specificity of the pp125FAK-β1 interaction and indicated that the pp125FAK and paxillin binding sites resided in the membrane proximal 13 residues of the β1 cytoplasmic domain.

The sequences of the cytoplasmic domains of the different β integrin subunits are quite divergent (Sastry and Horwitz, 1993). This diversity was exploited to further define residues required for binding to pp125FAK. pp125FAK was found to bind not only to a synthetic β1 peptide but also to synthetic peptides identical in sequence to the cyto-

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plasmic domains of the $\beta_1$ and $\beta_3$ integrin subunits (Fig. 3A). The $\beta_1$ and $\beta_3$ sequences are highly conserved throughout the membrane proximal region of the cytoplasmic domain and therefore binding to each was anticipated. The $\beta_1$ integrin sequence is strikingly different. Comparison of the sequences of $\beta_1$ and $\beta_2$ reveals a highly conserved aspartic acid and glutamic acid residue within the membrane proximal region of the cytoplasmic domain and these residues are highly conserved in other integrins as well (Fig. 3B; Sastry and Horwitz, 1993). Furthermore, this region of the cytoplasmic domain is predicted to form an $\alpha$ helix in which the aspartic acid and glutamic acid residues lie in close proximity on one face of the helix. To determine if these acidic residues were important in binding pp125$\text{FAK}$, a synthetic peptide similar to SP1 but with alanine residues substituted for the aspartic and glutamic acid residues was tested for binding activity. This variant peptide was unable to bind to pp125$\text{FAK}$ (Fig. 3C) or paxillin (data not shown). These results indicate that the highly conserved aspartic and glutamic acid residues are necessary for complex formation with pp125$\text{FAK}$, either by providing a negatively charged surface for binding or by influencing the conformation of the peptide to maintain the integrity of the binding site.

The sites of the $\beta_1$ peptide to which pp125$\text{FAK}$ and paxillin bind are virtually coincident, suggesting that binding of one might be dependent upon the other. This possibility is supported by our observations that pp125$\text{FAK}$ and paxillin can form a complex both in vivo and in vitro (Hildebrand et al., 1995). The interdependency of pp125$\text{FAK}$ and paxillin in integrin binding was examined by preclearing one of these proteins from CE cell lysates and testing for integrin binding activity of the other protein, which remained in the lysate. Immunodepletion of paxillin from cell lysates does not impair the $\beta_1$ binding activity of pp125$\text{FAK}$ (Fig. 4A). Similarly immunodepletion of pp125$\text{FAK}$ from CE cell lysates does not reduce the $\beta_1$ binding activity of paxillin (Fig. 4B). Thus the integrin binding activities of pp125$\text{FAK}$ and paxillin are independent.

The site on pp125$\text{FAK}$ to which the $\beta_1$ peptide binds was explored using extracts containing a naturally occurring variant of pp125$\text{FAK}$ called p41$\text{FRNK}$ (the COOH-terminal noncatalytic domain of pp125$\text{FAK}$ expressed from an alternately processed mRNA [Schafer et al., 1993]) or a series of engineered variants bearing deletions in the NH2- or COOH-terminal domains (Hildebrand et al., 1993). CE cells were transfected with replication competent retroviral vectors encoding each construct and upon full infection of the culture, lysates were prepared and incubated with $\beta_1$-beads. As above, ~10% of the pp125$\text{FAK}$ in the lysate bound to the $\beta_1$-beads. In contrast, virtually no p41$\text{FRNK}$ was retained on the beads (Fig. 5A). This observation was supported by the analysis of pp125$\text{FAK}$ deletion mutants. Deletion of virtually the entire COOH-terminal noncatalytic domain (corresponding to the sequences found in p41$\text{FRNK}$) did not compromise the $\beta_1$ binding activity of pp125$\text{FAK}$ (Fig. 5B). Thus the $\beta_1$ binding site within pp125$\text{FAK}$ does not reside within the COOH-terminal noncatalytic domain.

Since the $\beta_1$-binding site was not in the COOH-terminal domain, an in vitro translation approach was used to generate COOH-terminal truncations of pp125$\text{FAK}$ to further define the $\beta_1$ binding site of pp125$\text{FAK}$. An in vitro translated polypeptide containing residues 1–344 bound very efficiently to $\beta_1$ beads whereas in vitro translated p41$\text{FRNK}$ (corresponding to amino acids 693–1052 of pp125$\text{FAK}$) did not bind to $\beta_1$ beads (data not shown). To further test which domain mediated binding of pp125$\text{FAK}$ to $\beta_1$ beads,
domains of recombinant pp125FAK, expressed in E. coli, were tested for binding. An NH2-terminal fragment (residues 31-376) and a COOH-terminal fragment (residues 765-1052) of FAK were expressed as glutathione-S-transferase fusion proteins and purified on glutathione sepharose beads. The fusion proteins were labeled by phosphorylation with protein kinase A, which recognizes a sequence within the linker between the GST and FAK sequences. The FAK polypeptides were cleaved from the GST sequences with thrombin and the GST fragments, bound to the glutathione beads, and removed by centrifugation. The 32P-labeled fragments of pp125FAK were then incubated with β1 beads as above. As shown in Fig. 6, the NH2-terminal fragment of FAK efficiently bound to β1 beads, whereas the COOH-terminal fragment was incapable of binding to the β1 peptide. Furthermore the NH2-terminal fragment of pp125FAK efficiently bound to β1 but bound very poorly to the control peptide SPIAA (data not shown). These data demonstrate that pp125FAK directly binds to the β1 integrin cytoplasmic peptide and further supports the observation that a binding site resides within the NH2-terminal, noncatalytic domain.

Discussion

Like the integrins, a number of transmembrane receptors that are devoid of enzymatic activity trigger the phosphorylation of cellular proteins on tyrosine. In several instances this linkage is accomplished by the physical association of an intracellular protein tyrosine kinase with the cytoplasmic domain of the receptor. For example, the T cell surface markers CD4 and CD8 are physically associated via short cytoplasmic domains to the protein tyrosine kinase p56lk (Sefton, 1991). Similarly a number of receptors including the erythropoietin receptor and growth hormone receptor physically complex with the protein tyrosine kinase Jak2 (Ihle et al., 1994; Ziemecki et al., 1994). In each case binding of ligand to the receptor results in the activation of the catalytic activity of the associated kinase. The studies reported here provide evidence that integrin/pp125FAK signaling might occur in a similar manner.

Using the in vitro binding assay we have established that pp125FAK and the cytoskeletal protein paxillin can associate with a synthetic peptide modeled upon the COOH-terminal, cytoplasmic domain of the β1 integrin. These interactions are mediated by the membrane proximal region of the β1 cytoplasmic domain and two highly conserved, negatively charged residues which appear to be necessary for binding. Two scrambled peptides with an amino acid composition identical to SP1 were also tested for binding to pp125FAK. The scrambled peptides exhibited variable binding to pp125FAK. This is in direct contrast to SP1 which always bound to pp125FAK with high efficiency (data not shown). It is possible that this peptide is too short to be effectively scrambled, and that in scrambled form the peptide still exhibited sufficiently similar properties to SPI to bind to pp125FAK. It is unlikely, however, that the observed interaction is a nonspecific charge effect since pp125FAK and paxillin bind poorly to SP2. SP1 contains three basic and two acidic residues and a sequence of four consecutive charged amino acids (two positive and two negative). SP2 contains four basic and two acidic residues and likewise contains a sequence of four consecutive charged amino acids (two positive and two negative). Despite their similarity in charge the two peptides exhibit distinct binding specificities. Additional evidence for binding specificity comes from the analysis of FRNK binding. Although FRNK has been demonstrated to mediate extensive protein-protein interactions (Hildebrand et al., 1993, 1995), FRNK failed to bind to either β1- or SPI-peptide-containing beads.

It is interesting to note that there are two reports describing mutations of one or the other acidic residues within the SPI region of the β1 integrin. Variants with lesions at this site exhibit a small decrease in the capacity to localize to focal adhesions, but in combination with a second point mutation in the membrane distal portion of the β1 cytoplasmic domain exhibits a severe reduction in localization to focal adhesions (Reszka et al., 1992). These acidic residues have also been altered in the β1 integrin subunit of Drosophila melanogaster (Grinblat et al., 1993). This variant has been substituted for the wild-type allele in vivo and the resulting flies exhibit a defect in the normal development of the wing. These results implicate this region of the integrin as a critical region for the normal function of these receptors in the cell.

Recent evidence strongly supports the functional linkage of pp125FAK to integrins containing the β1 or β3 subunits (Guan and Shalloway, 1992; Lipfert et al., 1992). Using chimeric receptors containing extracellular and transmembrane sequences from the IL-2-receptor fused to the cytoplasmic domains of various integrins it has been established that cross-linking of the tails of β integrin subunits alone is sufficient to induce tyrosine phosphorylation (Akiyama et al., 1994). This analysis has also established that the cytoplasmic domains of the β3, β2, and β3 integrin subunits can trigger tyrosine phosphorylation, but the tail of the β3 variant integrin subunit cannot. Exogenous expression of human β3 integrin subunits in CHO cells and induction of clustering by plating onto immobilized anti-human β3 monoclonal antibodies indicates that the β3 subunit is also unable to activate PTKs (Balzac et al., 1994). Comparison of the sequences of the β1, β3, and β5 integrin subunits reveals

**Figure.** 6. pp125FAK binds directly to β1 beads. Glutathione-S-transferase fusion proteins containing FAK residues 31-376 (n-term-lanes 1 and 2) or 765-1052 (c-term-lanes 3 and 4) were expressed in E. coli, purified and labeled as in Materials and Methods. Each was tested for its ability to bind β1 beads (lanes 2 and 4) and bound material analyzed by SDS-PAGE and autoradiography. Loading control (10% of the material incubated with the β1 beads) is shown in lanes 1 and 3.
extensive homology in the membrane proximal region and divergence in sequence towards the carboxy terminus with but a few highly conserved charged residues in this region. The sequences for the $\beta_1$ and $\beta_3$ integrin subunits diverge from the $\beta_1$ and $\beta_3$ sequences in the region which is poorly conserved between these integrins. It is therefore possible that the few scattered conserved residues in the membrane distal region of the cytoplasmic tail of the $\beta$ integrin subunits are linked to the signaling apparatus that activates PTKs. Alternatively, the highly conserved, membrane proximal sequences of the cytoplasmic domains may regulate the activation of PTKs, but the distal sequences may be required for some other facet of signaling, for example tethering the signaling complex in the proper cellular location to transduce the signal.

The in vitro binding data presented above are consistent with a model in which pp125$^{\text{FAK}}$ may be physically associated with the cytoplasmic domain of $\beta_1$ and $\beta_3$ integrins within the cell. Evidence supporting this hypothesis has come from two different experimental approaches. First, capping of cell surface integrins can, under certain circumstances, lead to capping of many focal adhesion-associated proteins. Cross-linking of integrins on human fibroblasts using certain anti-$\beta_1$ or anti-$\alpha_1$ antibodies leads to specific capping of a very limited subset of focal adhesion-associated proteins including pp125$^{\text{FAK}}$ (Miyamoto et al., 1995). Secondly, analysis of a series of $\beta_3$ cytoplasmic domain deletion variants revealed that the membrane proximal 12 amino acids of the $\beta_3$ integrin are sufficient to trigger $\alpha_{13}\beta_3$-dependent phosphorylation of pp125$^{\text{FAK}}$ on tyrosine (S. Shattil and M. Ginsberg, personal communication). Thus the $\beta_3$ subunit sequences that are sufficient to induce the phosphorylation of pp125$^{\text{FAK}}$ in vivo coincides with the pp125$^{\text{FAK}}$-binding site in vitro.

Despite the fact that similar integrin sequences are implicated in binding to both pp125$^{\text{FAK}}$ and paxillin, and despite our observation that pp125$^{\text{FAK}}$ and paxillin physically associate (Hildebrand et al., 1995), our data indicate that binding of these two cytoskeletal proteins to $\beta_3$ occurs independently. Firstly, pp125$^{\text{FAK}}$ can bind directly to the $\beta_3$ peptide via sequences within its NH$_2$-terminal noncatalytic domain. Secondly, variants of pp125$^{\text{FAK}}$ that fail to associate with paxillin (e.g., d1686-1011) still exhibit $\beta_3$ binding activity. Thirdly, depletion of paxillin from CE lysates by immunoprecipitation does not impair binding of pp125$^{\text{FAK}}$ to the $\beta_3$ peptide. Fourthly, immunodepletion of pp125$^{\text{FAK}}$ from CE lysates does not impair binding of paxillin to the $\beta_3$ peptide. Finally, 10-fold overexpression of pp125$^{\text{FAK}}$ in CE lysates, resulting in a 10-fold increase in the absolute amount of pp125$^{\text{FAK}}$ that binds to $\beta_3$ beads, does not result in an increase in the amount of paxillin that binds $\beta_3$ beads (data not shown). Whether paxillin binding to $\beta_3$ is direct or indirect is presently an unresolved issue that will require reconstitution experiments using purified recombinant paxillin to adequately address.

Although we have shown complex formation between the $\beta_3$ integrin subunit and pp125$^{\text{FAK}}$ in vitro, this interaction is unlikely to be a major determinant in directing the localization of pp125$^{\text{FAK}}$ to cellular focal adhesions in vivo. p41FRNK, the autonomously expressed COOH-terminal domain of pp125$^{\text{FAK}}$, localizes to focal adhesions (Schaller et al., 1993) yet fails to bind to $\beta_3$ beads. Furthermore, several COOH-terminal deletion mutants of pp125$^{\text{FAK}}$, which are defective for focal adhesion localization (Hildebrand et al., 1993), bind efficiently to the $\beta_3$ peptide in vitro. Therefore it appears that pp125$^{\text{FAK}}$ is targeted to the focal adhesion by binding to a protein other than the $\beta_3$ integrin, the identity of which is currently unknown.

The precise mechanism by which FAK becomes activated by the integrin remains speculative. Binding of ligand might induce a conformational change in the cytoplasmic domains of the $\alpha$-$\beta$ integrin dimer. This conformational change could expose the pp125$^{\text{FAK}}$ binding site triggering complex formation and the activation of pp125$^{\text{FAK}}$ by occupancy of its integrin binding site. This model predicts that pp125$^{\text{FAK}}$ will always be active when complexed with $\beta_1$ and that the key regulatory event is the formation of this complex. Alternatively, pp125$^{\text{FAK}}$ may bind constitutively to $\beta_3$ integrin subunits. Ligand induced conformational changes in the integrin may in turn induce a conformational change in pp125$^{\text{FAK}}$ resulting in its activation. In both of these models integrin clustering is not necessary for the activation of pp125$^{\text{FAK}}$. However, integrin clustering, induced by ligand binding, could potentially play a very important role in the activation of pp125$^{\text{FAK}}$. Integrin clustering may directly cause pp125$^{\text{FAK}}$ clustering, leading to the activation of pp125$^{\text{FAK}}$ by a mechanism that is analogous to the activation of the growth factor receptor protein tyrosine kinases. These enzymes become activated by ligand-induced dimerization and subsequent transphosphorylation (Ullrich and Schlessinger, 1990). Whether transphosphorylation of pp125$^{\text{FAK}}$ will be an important facet of its activation remains to be tested.

Current models of the structure of focal adhesions portray the integrins as key elements that bind to extracellular matrix and simultaneously anchor a complex array of structural proteins of the cytoskeleton. Our observations that signaling molecules may also physically associate with the integrins suggest that multi-component signaling complexes may form in parallel to structural complexes at sites of integrin adhesion. Elucidation of the interactions of these signaling molecules with each other and the integrins will be the key to understanding signal transduction through the integrins.

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