Focal Adhesion Kinase and Paxillin Bind to Peptides Mimicking β 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^{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^{FAK}. Paxillin physically complexes with two protein tyrosine kinases, pp60^{src} and Csk (COOH-terminal src kinase), and the oncoprotein p47$^{gag\text{-}crk}$, 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 β_1 integrin can bind to paxillin and pp125^{FAK} from chicken embryo cell lysates. The NH_2-terminal, noncatalytic domain of pp125^{FAK} can bind directly to the cytoplasmic tail of β_1 and recognizes integrin sequences distinct from those involved in binding to α-actinin. Paxillin binding is independent of pp125^{FAK} binding despite the fact that both bind to the same region of β_1. These results demonstrate that the cytoplasmic domain of the β 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 $[Ca^{2+}]_i$ (Richter et al., 1990; Jaconi et al., 1991; Pelletier et al., 1992; Schwartz, 1993) and pH_i (Schwartz et al., 1991*a*,*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),[1] pp125^{FAK} (Guan and Shalloway, 1992; Lipfert et al., 1992). pp125^{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^{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^{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^{src} and src-related PTKs physically associate with pp125^{FAK} through interactions mediated by the SH2 domains within the src-related PTKs and the major site of autophosphorylation of pp125^{FAK}, tyrosine^{397} (Cobb et al., 1993; Schaller et al., 1994). The recruitment of src-like PTKs into pp125^{FAK} containing complexes may be a mechanism for amplification and/or diversification of the molecular signals that trigger the activation of pp125^{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 α-actinin (Otey et al., 1990), which binds directly to actin (Bennett et al., 1984). Integrins also bind to talin (Horwitz et al., 1986;

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1. *Abbreviations used in this paper*: CE, chicken embryo; Csk, COOH-terminal src kinase; FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; GST, glutathione-S-transferase; PTK, protein tyrosine kinase; SH2 domain, src-homology 2 domain; SH3 domain, src-homology 3 domain; SP1, short peptide 1.

Tapley et al., 1989), which binds to vinculin (Burridge and Mangeat, 1984). Vinculin in turn, complexes with α-actinin (Belkin and Koteliansky, 1987; Wachsstock 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 $pp60^{src}$ (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 $pp60^{src}$ (Weng et al., 1993), Csk (COOH-terminal src kinase; a PTK that phosphorylates and negatively regulates the activity of $pp60^{c\text{-}src}$) (Sabe et al., 1994) and the adaptor protein $p47^{gag\text{-}crk}$ (Birge et al., 1993). Csk and $p47^{gag\text{-}crk}$ bind to tyrosine-phosphorylated motifs on paxillin via their src-homology 2 (SH2) domains, whereas $pp60^{src}$ 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, $pp125^{FAK}$. We have considered several mechanisms by which the integrins might regulate the activity of $pp125^{FAK}$ and have tested the hypothesis that $pp125^{FAK}$ is an integrin binding protein. Using an in vitro assay we have established that the membrane proximal region of the cytoplasmic domain of the β_1 integrin subunit binds directly to the NH_2-terminal region of $pp125^{FAK}$. The same fragment of the β_1 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 β_1 (CKLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK), β_3 (CKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT), SP1 (CKLLMIIHDRREFA), SP2 (CFAKFEKEKMNAKW), SP3 (CKWDTGENPIYKSA), and SP4 (CAVTTVVNPKYEGK) were synthesized by the Protein Chemistry Laboratory at the University of North Carolina at Chapel Hill. SP1 was also purchased from Quality Controlled Biochemicals (Hopkinton, MA) as was peptide SP1AA (CKLLMIIHARRAFA). The β_2 peptide (CKALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES) was a gift of Dr. Fredrick Pavalko. Mass spectrometry and amino acid analysis was carried out to confirm the sequence and purity of individual peptides. Each was coupled via its NH_2-terminal cysteine residue to thiolpropyl–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 = 8.08×10^3 $M^{-1} \cdot cm^{-1}$ 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 engineered to express either wild type $pp125^{FAK}$ or one of a number of variants (Hildebrand et al., 1993; Schaller et al., 1993) were lysed with modified radioimmunoprecipitation assay buffer as described (Kanner et al., 1989). Lysates were diluted to 500 μg/ml and ~500 μg of total protein was incubated with peptide coated sepharose beads at 4°C for 1–2 h with constant rocking. For competition experiments 3 or 6 mM free β_1 peptide was incubated with the lysate for 1 h before the addition of the β_1-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-$pp125^{FAK}$ 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-vinculin mAb Vin 11-5 (Sigma Chem. Co., St. Louis, MO), an anti–α-actinin serum (a gift of Dr. Keith Burridge, 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 $pp125^{FAK}$ 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 En^{3}hance (Dupont, Boston, MA).

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 $pp125^{FAK}$ 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 sequences was phosphorylated in vitro (Kaelin 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 aliquot of the cleared lysate was removed as loading control and the remainder diluted to 1 ml with radioimmunoprecipitation assay buffer, incubated with β_1 beads for 1 h, washed, and analyzed as above.

Results

To test the hypothesis that $pp125^{FAK}$ 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 β_1 integrin subunit, coupled to thiopropyl-sepharose beads. The beads were incubated with lysates prepared from either CE cells or CE cells overexpressing $pp125^{FAK}$, recovered by centrifugation and extensively washed. Proteins bound to the beads were analyzed by Western blotting (Fig. 1 *A*). 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 $pp125^{FAK}$ in the lysates bound to the β_1 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 β_1 beads (Fig. 1 *A*). In fact a similar proportion of α-actinin (~10%), a known integrin binding protein, was also retained on the β_1 beads (Fig. 1 *A*). Preincubation of the lysate with free peptide effectively inhibited binding of pp125^{FAK} to the β_1 peptide coated beads, indicating that pp125^{FAK} 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 pp125^{FAK}) were incubated with beads and binding of specific proteins was monitored by Western blotting. Vinculin exhibited a small amount of binding to β_1-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 β_1 integrin.

The binding of cytoskeletal proteins to the β_1 peptide sequence was further characterized using four shorter peptides, each corresponding to approximately one-quarter of the full- length β_1 peptide (Otey et al., 1993). Incubation of each of the peptides, coupled to beads, with cell lysates revealed that pp125^{FAK} 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*). pp125^{FAK} 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 pp125^{FAK}, FAK-related nonkinase (FRNK), failed to bind to SP1 (Fig. 2 *D*). These results further underscored the specificity of the pp125^{FAK}-β_1 interaction and indicated that the pp125^{FAK} and paxillin binding sites resided in the membrane proximal 13 residues of the β_1 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 pp125^{FAK}. pp125^{FAK} was found to bind not only to a synthetic β_1 peptide but also to synthetic peptides identical in sequence to the cyto-

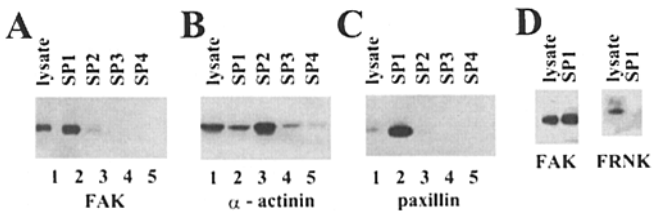

Figure 2. pp125^{FAK} and paxillin bind to SP1. Approximately 500 μg of CE cell lysate was incubated with beads coupled to SP1 (lane *2*), SP2 (lane *3*), SP3 (lane *4*), or SP4 (lane *5*), the beads washed and bound proteins subjected to Western blotting to detect pp125^{FAK} (*A*), α-actinin (*B*), paxillin (*C*) or FAK or FRNK (*D*). Lane *1* contains 50 μg of lysate as a loading control.

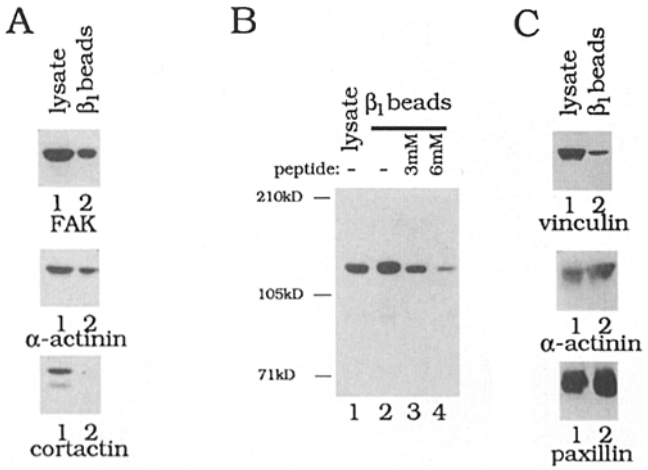

Figure 1. pp125^{FAK} and paxillin complex with β_1 peptide. (*A*) Approximately 500 μg of lysate from CE cells overexpressing pp125^{FAK} were incubated with β_1 beads, washed, and subjected to Western blotting analysis (lane *2*). A loading control, 10% or 50 μg of lysate was analyzed in parallel (lane *1*). Blots were probed with BC3 (*top*) to detect pp125^{FAK}, a polyclonal antiserum directed against α-actinin (*middle*) or mAb 4F11 to detect cortactin (*bottom*). (*B*) pp125^{FAK} was bound to β_1 beads and analyzed as in *A* (lanes *1* and *2*). Alternatively, lysates were preincubated with 3 (lane *3*) or 6 mM (lane *4*) free β_1 peptide prior to incubation with β_1 beads. (*C*) Approximately 500 μg of CE cell lysate was incubated with β_1 beads and the bound proteins analyzed by Western blotting (lane *2*). 50 μg of lysate was run as a loading control (lane *1*). The blots were probed with mAb Vin 11-5 to detect vinculin (*top*), antiserum directed against α-actinin (*middle*) or mAb directed against paxillin (*bottom*).

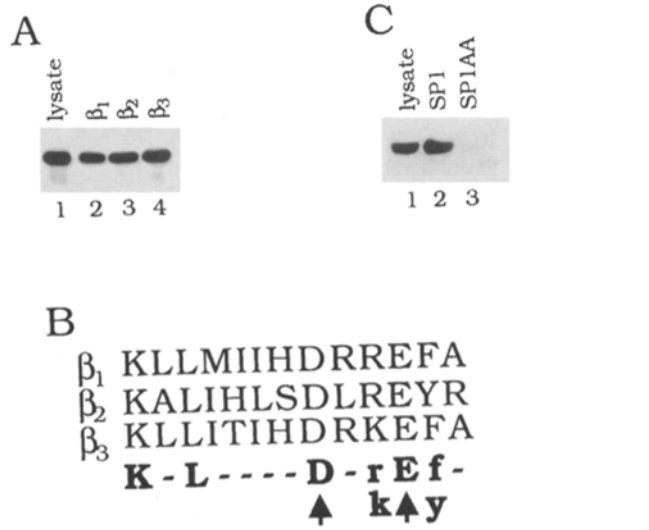

Figure 3. pp125^{FAK} binds to β_1, β_2, and β_3 integrin peptides. (*A*) Lysate from CE cells expressing exogenous pp125^{FAK} was incubated with β_1 beads (lane *2*), β_2 beads (lane *3*), or β_3 beads (lane *4*) and the bound proteins eluted, subjected to SDS-PAGE and Western blotted to detect pp125^{FAK}. Loading control is shown in lane *1*. (*B*) Sequence comparison of the β_1, β_2, and β_3 integrin peptides within the region corresponding to SP1. A consensus sequence is shown in bold. Arrows denote highly conserved acidic residues. (*C*) Lysate from CE cells expressing exogenous pp125^{FAK} was incubated with SP1 beads (lane *2*) or SP1AA beads (lane *3*) and the bound proteins blotted for pp125^{FAK}. Loading control is in lane *1*.

plasmic domains of the β_2 and β_3 integrin subunits (Fig. 3 *A*). The β_1 and β_3 sequences are highly conserved throughout the membrane proximal region of the cytoplasmic domain and therefore binding to each was anticipated. The β_2 integrin sequence is strikingly different. Comparison of the sequences of β_1 and β_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. 3 *B*; Sastry and Horwitz, 1993). Furthermore, this region of the cytoplasmic domain is predicted to form an α 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^{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^{FAK} (Fig. 3 *C*) or paxillin (data not shown). These results indicate that the highly conserved aspartic and glutamic acid residues are necessary for complex formation with pp125^{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 β_1 peptide to which pp125^{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^{FAK} and paxillin can form a physical complex both in vivo and in vitro (Hildebrand et al., 1995). The interdependency of pp125^{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 β_1 binding activity of pp125^{FAK} (Fig. 4 *A*). Similarly immunodepletion of pp125^{FAK} from CE cell lysates does not reduce the β_1 binding activity of paxillin (Fig. 4 *B*). Thus the integrin binding activities of pp125^{FAK} and paxillin are independent.

The site on pp125^{FAK} to which the β_1 peptide binds was explored using extracts containing a naturally occurring variant of pp125^{FAK} called p41^{FRNK} (the COOH-terminal noncatalytic domain of pp125^{FAK} expressed from an alternately processed mRNA [Schaller et al., 1993]) or a series of engineered variants bearing deletions in the NH_2- 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 β_1-beads. As above, ~10% of the pp125^{FAK} in the lysate bound to the β_1-beads. In contrast, virtually no p41^{FRNK} was retained on the beads (Fig. 5 *A*). This observation was supported by the analysis of pp125^{FAK} deletion mutants. Deletion of virtually the entire COOH-terminal noncatalytic domain (corresponding to the sequences found in p41^{FRNK}) did not compromise the β_1 binding activity of pp125^{FAK} (Fig. 5 *B*). Thus the β_1 binding site within pp125^{FAK} does not reside within the COOH-terminal noncatalytic domain.

Since the β_1-binding site was not in the COOH-terminal domain, an in vitro translation approach was used to generate COOH-terminal truncations of pp125^{FAK} to further define the β_1 binding site of pp125^{FAK}. An in vitro translated polypeptide containing residues 1–344 bound very efficiently to β_1 beads whereas in vitro translated p41^{FRNK} (corresponding to amino acids 693–1052 of pp125^{FAK}) did not bind to β_1 beads (data not shown). To further test which domain mediated binding of pp125^{FAK} to β_1 beads,

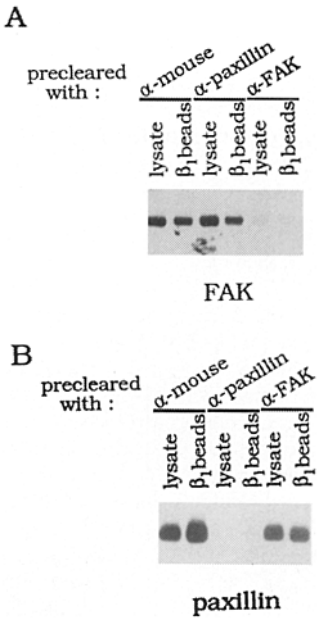

Figure 4. pp125^{FAK} and paxillin bind independently to β_1 peptide. CE cell lysate was immunodepleted by incubation with an anti-paxillin mAb or an anti-pp125^{FAK} mAb (2A7). A control immunoprecipitation was performed using goat anti–mouse antibodies (α-*mouse*). An aliquot equal to 10% of the total volume was removed from the depleted supernatants as a loading control (*lysate*) and the remainder incubated with β_1 beads (β_1). Proteins that bound to the beads were detected by Western blotting using anti-pp125^{FAK} rabbit serum, BC3 (*A*) or anti-paxillin mAb (*B*).

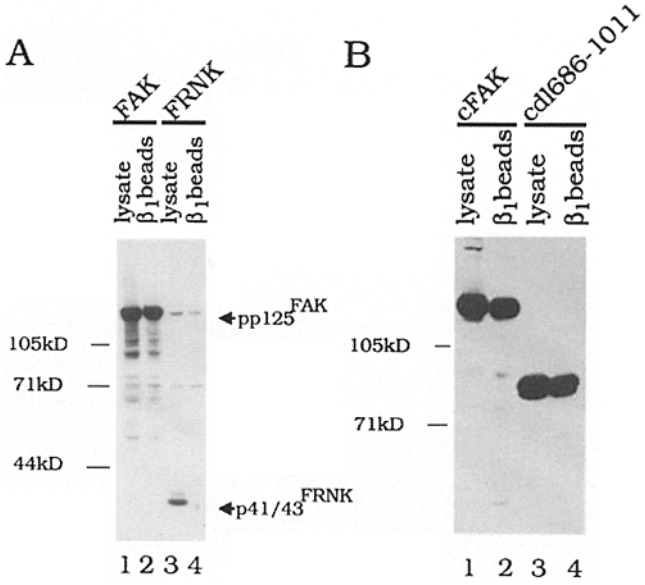

Figure 5. pp125^{FAK} does not bind via its COOH terminus. (*A*) Lysates from CE cells overexpressing pp125^{FAK} (lanes *1* and *2*) or p41^{FRNK} (lanes *3* and *4*) were incubated with β_1 beads (lanes *2* and *4*) and the bound proteins analyzed by Western blotting for pp125^{FAK}. Loading controls are in lanes *1* and *3*. (*B*) Lysates from CE cells expressing an epitope-tagged derivative of wild type pp125^{FAK} (lanes *1* and *2*) or epitope-tagged d1686-1011 (lanes *3* and *4*) were incubated with β_1 beads (lanes *2* and *4*) and the bound proteins analyzed by blotting with mAb KT3 to detect the tag. Loading controls are in lanes *1* and *3*.

domains of recombinant pp125^{FAK}, expressed in *E. coli*, were tested for binding. An NH_2-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 ^{32}P-labeled fragments of pp125^{FAK} were then incubated with β_1 beads as above. As shown in Fig. 6, the NH_2-terminal fragment of FAK efficiently bound to β_1 beads, whereas the COOH-terminal fragment was incapable of binding to the β_1 peptide. Furthermore the NH_2-terminal fragment of pp125^{FAK} efficiently bound to SP1 but bound very poorly to the control peptide SP1AA (data not shown). These data demonstrate that pp125^{FAK} directly binds to the β_1 integrin cytoplasmic peptide and further supports the observation that a binding site resides within the NH_2-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 p56^{lck} (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; Ziemiecki 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/pp125^{FAK} signaling might occur in a similar manner.

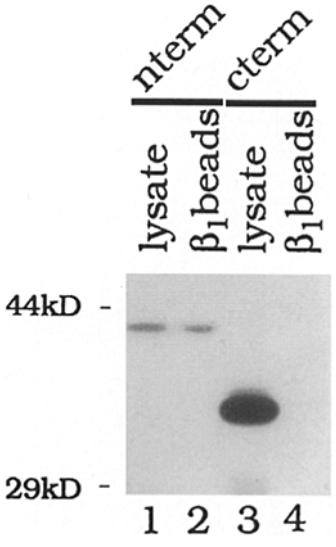

Figure. 6. pp125^{FAK} binds directly to β_1 beads. Glutathione-S-transferase fusion proteins containing FAK residues 31–376 (nterm-lanes *1* and *2*) or 765–1052 (cterm-lanes *3* and *4*) were expressed in *E. coli*, purified and labeled as in Materials and Methods. Each was tested for its ability to bind β_1 beads (lanes *2* and *4*) and bound material analyzed by SDS-PAGE and autoradiography. Loading control (10% of the material incubated with the β_1 beads) is shown in lanes *1* and *3*.

Using the in vitro binding assay we have established that pp125^{FAK} and the cytoskeletal protein paxillin can associate with a synthetic peptide modeled upon the COOH-terminal, cytoplasmic domain of the β_1 integrin. These interactions are mediated by the membrane proximal region of the β_1 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 pp125^{FAK}. The scrambled peptides exhibited variable binding to pp125^{FAK}. This is in direct contrast to SP1 which always bound to pp125^{FAK} 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 SP1 to bind to pp125^{FAK}. It is unlikely, however, that the observed interaction is a nonspecific charge effect since pp125^{FAK} 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 β_1- or SP1-peptide–containing beads.

It is interesting to note that there are two reports describing mutations of one or the other acidic residues within the SP1 region of the β_1 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 β_1 cytoplasmic domain exhibits a severe reduction in localization to focal adhesions (Reszka et al., 1992). These acidic residues have also been altered in the β_1 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 pp125^{FAK} to integrins containing the β_1 or β_3 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 β_1, β_3, and β_5 integrin subunits can trigger tyrosine phosphorylation, but the tail of the β_{3B} variant integrin subunit cannot. Exogenous expression of human β_1 integrin subunits in CHO cells and induction of clustering by plating onto immobilized anti-human β_1 monoclonal antibodies indicates that the β_{1B} subunit is also unable to activate PTKs (Balzac et al., 1994). Comparison of the sequences of the β_1, β_3, and β_5 integrin subunits reveals

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 β_{1B} and β_{3B} integrin subunits diverge from the β_1 and β_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 β 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^{FAK} may be physically associated with the cytoplasmic domain of β_1 and β_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 cocapping of many focal adhesion-associated proteins. Cross-linking of integrins on human fibroblasts using certain anti-β_1 or anti-α_1 antibodies leads to specific cocapping of a very limited subset of focal adhesion-associated proteins including pp125^{FAK} (Miyamoto et al., 1995). Secondly, analysis of a series of β_3 cytoplasmic domain deletion variants revealed that the membrane proximal 12 amino acids of the β_3 integrin are sufficient to trigger $\alpha_{IIb}\beta_3$-dependent phosphorylation of pp125^{FAK} on tyrosine (S. Shattil and M. Ginsberg, personal communication). Thus the β_3 subunit sequences that are sufficient to induce the phosphorylation of pp125^{FAK} in vivo coincides with the pp125^{FAK}-binding site in vitro.

Despite the fact that similar integrin sequences are implicated in binding to both pp125^{FAK} and paxillin, and despite our observation that pp125^{FAK} and paxillin physically associate (Hildebrand et al., 1995), our data indicate that binding of these two cytoskeletal proteins to β_1 occurs independently. Firstly, pp125^{FAK} can bind directly to the β_1 peptide via sequences within its NH_2-terminal noncatalytic domain. Secondly, variants of pp125^{FAK} that fail to associate with paxillin (e.g., d1686-1011) still exhibit β_1 binding activity. Thirdly, depletion of paxillin from CE lysates by immunoprecipitation does not impair binding of pp125^{FAK} to the β_1 peptide. Fourthly, immunodepletion of pp125^{FAK} from CE lysates does not impair binding of paxillin to the β_1 peptide. Finally, 10-fold overexpression of pp125^{FAK} in CE lysates, resulting in a 10-fold increase in the absolute amount of pp125^{FAK} that binds to β_1 beads, does not result in an increase in the amount of paxillin that binds β_1 beads (data not shown). Whether paxillin binding to β_1 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 β_1 integrin subunit and pp125^{FAK} in vitro, this interaction is unlikely to be a major determinant in directing the localization of pp125^{FAK} to cellular focal adhesions in vivo. p41^{FRNK}, the autonomously expressed COOH-terminal domain of pp125^{FAK}, localizes to focal adhesions (Schaller et al., 1993) yet fails to bind to β_1 beads. Furthermore, several COOH-terminal deletion mutants of pp125^{FAK}, which are defective for focal adhesion localization (Hildebrand et al., 1993), bind efficiently to the β_1 peptide in vitro. Therefore it appears that pp125^{FAK} is targeted to the focal adhesion by binding to a protein other than the β_1 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 α-β integrin dimer. This conformational change could expose the pp125^{FAK} binding site triggering complex formation and the activation of pp125^{FAK} by occupancy of its integrin binding site. This model predicts that pp125^{FAK} will always be active when complexed with β_1 and that the key regulatory event is the formation of this complex. Alternatively, pp125^{FAK} may bind constitutively to β integrin subunits. Ligand induced conformational changes in the integrin may in turn induce a conformational change in pp125^{FAK} resulting in its activation. In both of these models integrin clustering is not necessary for the activation of pp125^{FAK}. However, integrin clustering, induced by ligand binding, could potentially play a very important role in the activation of pp125^{FAK}. Integrin clustering may directly cause pp125^{FAK} clustering, leading to the activation of pp125^{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^{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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