Direct Association of pp125^{FAK} with Paxillin, the Focal Adhesion–targeting Mechanism of pp125^{FAK}

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Summary

Focal adhesion kinase (pp125^{FAK}) is localized to focal adhesions and tyrosine phosphorylated by the engagement of β1 integrins. However, it is unclear how pp125^{FAK} is linked to integrin molecules. We demonstrate that pp125^{FAK} is directly associated with paxillin, a 68-kD cytoskeleton protein. The COOH-terminal domain of pp125^{FAK} spanning FAK residues 919–1042 is sufficient for paxillin binding and has vinculin-homologous amino acids, which are essential for paxillin binding. Microinjection and subsequent immunohistochemical analysis reveal that glutathione *S*-transferase–FAK fusion proteins, which bind to paxillin, localize to focal adhesions, whereas fusion proteins with no paxillin-binding activity do not localize to focal adhesions. These findings strongly suggest that pp125^{FAK} is localized to focal adhesions by the direct association with paxillin.

β1 integrins, also known as very late activation antigens, constitute a subfamily of integrin adhesion receptors that is comprised by at least nine α subunits sharing a common β1 subunit (1, 2). β1 integrins function as cell-surface receptors for extracellular matrix protein (ECM)[1] and also mediate cell-to-cell interaction (1, 2). The marked changes in morphology and behavior that occur when cells interact with ECM through their integrins suggest that these receptors can function as transducers of extracellular signals into cells (2, 3). Several laboratories including ours have shown that β1 integrins synergize with the TCR–CD3 pathway to promote T cell proliferation (4–6). β1 integrins are also reported to be involved in T or B cell differentiation through interaction with fibronectin expressed by stromal cells in thymus or bone marrow, respectively (7, 8). Furthermore, it was also reported that integrin–ECM binding induced increases of cytoplasmic Ca^{2+} and cytoplasmic pH (9, 10). These facts strongly support the notion that the interaction between β1 integrins and ECM transmits signals into the interior of cells.

In an effort to clarify mechanisms of T cell costimulatory signals mediated by β1 integrins, we found that tyrosine kinase activation might be a key event in this process. We showed that tyrosine phosphorylation of 105–130-kD proteins was induced by the ligation of β1 or α4 integrin subunits using either fibronectin (FN) or mAbs in human peripheral T cells and H9, a T lymphoblastoid cell line (11, 12). Several independent studies also showed that tyrosine phosphorylation of proteins in the range of 120–130 kD is induced by the engagement of β1 integrins in different types of cells including mouse fibroblasts and human epidermal cancer cell line (13, 14). One of these tyrosine-phosphorylated proteins was identified as the tyrosine kinase, focal adhesion kinase (pp125^{FAK}) (15, 16).

pp125^{FAK} was first identified as a putative substrate for the oncogenic retrovirus gene product pp60$^{v\text{-}src}$ (17). Deduced amino acid sequence revealed FAK to be a novel cytoplasmic tyrosine kinase having relatively low homology to other known kinases and lacking SH2 and SH3 domains (15). pp125^{FAK} is localized to focal adhesions (15), which are the sites where cells adhere to the substrata via integrin–ECM binding. Subsequently, pp125^{FAK} was shown to be tyrosine phosphorylated by the ligation of β1 integrins (18, 19). Since integrins lack an intrinsic protein tyrosine kinase (PTK) activity, these findings about pp125^{FAK} strongly suggest important roles of pp125^{FAK} in the β1 integrin–mediated signaling. However, the nature of the interaction between β1 integrins and pp125^{FAK} remains unclear.

FAK-related nonkinase (FRNK), an autonomously expressed COOH-terminal non-catalytic domain of pp125^{FAK}, was reported to be localized to focal adhesions (20), suggesting that pp125^{FAK} COOH-terminal region plays an important role in the focal adhesion targeting (FAT) of pp125^{FAK}. Moreover, Hildebrand et al. reported that the FAT domain of pp125^{FAK}, which is responsible for pp125^{FAK}

[1] *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; ECM, extracellular matrix protein; FAT, focal adhesion targeting; FN, fibronectin; FRNK, FAK-related nonkinase; GST, glutathione *S*-transferase; PBS-T, PBS containing 0.1% Tween 20; PBS1, paxillin-binding subdomain 1; PBS2, paxillin-binding subdomain 2; pp125^{FAK}, focal adhesion kinase; PTK, protein tyrosine kinase.

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localization to focal adhesions, is located in the COOH-terminal region of pp125^{FAK} (21). pp125^{FAK} and FRNK were reported to be coprecipitated with paxillin, a 68-kD cytoskeletal protein that is localized to focal adhesions (22, 23). However, to date, the precise association of pp125^{FAK} via its FAT domain with other molecules and the functional consequence of such association remain unresolved.

In this study, we demonstrate that pp125^{FAK} is directly associated with paxillin, yet tyrosine phosphorylations of pp125^{FAK} and paxillin are not necessary for this association. The paxillin-binding domain of pp125^{FAK} that we have identified is homologous to the paxillin-binding domain of vinculin (24). Mutations in the conserved amino acid residues between pp125^{FAK} and vinculin result in the loss of paxillin-binding activity. The relevance between paxillin-binding activity and FAT of pp125^{FAK} is presented by using various deletion and substitution mutants of pp125^{FAK}.

Materials and Methods

Cell Culture and Stimulation. A human T lymphoblastoid cell line, HPB-ALL, a human breast cancer cell line, T-47D, and 3T3 Swiss albino cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in 7.5% CO_2 incubator supplemented with 10% newborn calf serum in RPMI 1640, 10% FCS in RPMI 1640, and 5% FCS in DMEM. For stimulation, HPB-ALL cells were washed three times with Iscove's serum-free medium (Sigma Chemical Co., St. Louis, MO), incubated for 30 min in CO_2 incubator in plates coated with 5 μg/ml human fibronectin (GIBCO BRL, Gaithersburg, MD) or antibodies, and blocked with 1% BSA (Fraction V; Sigma Chemical Co.). After incubation, cells attached to substrata were lysed on plates in 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM sodium pyrophosphate, 1% NP-40, 20 mM iodoacetamide, 0.2 trypsin inhibitor U/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, and 0.4 mM sodium orthovanadate). Cells not attached to substrata were collected by centrifugation and lysed in the same lysis buffer used for on-plate lysis. T-47D cells were washed twice with PBS and lysed in 1% NP-40 lysis buffer.

cDNA Cloning of Human FAK Gene and Construction of Glutathione S-Transferase (GST)–FAK Fusion Protein Expression Vectors. Human FAK cDNA was obtained by screening λgt 10 human fetal brain cDNA library (CLONTECH, Palo Alto, CA) with ^{32}P-labeled chick FAK cDNA fragments, which were kindly provided by J. T. Parsons (University of Virginia, Charlottesville, VA). 12 cDNA clones of various lengths were isolated. Full-length FAK cDNA reconstructed from three cDNA clones carried 4,347 bp in total and an open-reading frame coding 1,082 amino acids. This open-reading frame carried 30 extra amino acids in the NH_2-terminal region in addition to reported human or mouse FAK cDNAs (18, 25). In this article, the number of FAK amino acid indicated the number of 1,052 amino acids open-reading frame for the convenience of readers to compare with other publications.

Plasmids that coded GST fusion proteins were constructed by the insertion of human FAK cDNA fragments into pGEX-2T or pGEX-3X (Pharmacia Biotech Inc., Piscataway, NJ). Insert DNA fragments for deletion mutants were developed by appropriate restriction enzymes or by PCR with specific primers flanked with BamHI site (5′ primer) and with EcoRI site (3′ primer). Substitution mutants were generated by PCR to substitute one amino acid residue of the GST-FAK fusion protein containing residues 896–1052 (26). JM 105 cells carrying plasmids were inoculated in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside to express GST-FAK fusion proteins. Cells were collected by centrifuge, resuspended in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), sonicated, and incubated with BSA-blocked glutathione-Sepharose 4B (Pharmacia Biotech. Inc.). After incubation, beads were washed with NETN buffer and subjected to precipitation.

Immunoprecipitation and Immunoblotting. To establish 10G2 mAb, the GST-FAK fusion protein containing FAK residues 706–1052 was used as the antigen and the source for screening hybridoma. Culture supernatant or ascites of 10G2 mAb were purified by protein G (Pierce, Rockford, IL).

For immunoprecipitation, cells were lysed in 1% NP-40 lysis buffer. Cellular lysates were incubated overnight with rotation in the cold room with glutathione-Sepharose conjugated with GST-FAK fusion proteins or antiphosphotyrosine mAb–conjugated beads (4G10, Upstate Biotechnology, Inc., Lake Placid, NY). Otherwise, cellular lysates were incubated overnight with first antibodies and then with goat anti–mouse IgG Ab–conjugated beads (Sigma Chemical Co.) for 4 h with rotation in the cold room. Beads were washed with 1% NP-40 washing buffer (1% NP-40, 50 mM Tris-HCl, pH 8.0, 140 mM NaCl, and 2.5 mM EDTA) five times. Washed beads were boiled 5 min at 100°C in the presence of 2% SDS and 0.1 M dithiothreitol, and supernatants of boiled samples were loaded onto SDS-polyacrylamide gels. For the second immunoprecipitation, washed beads were boiled 5 min at 100°C in the presence of 1% SDS, and the supernatants were precipitated with antibodies in the 1% NP-40 washing buffer containing 0.1% SDS.

For immunoblotting, samples were subjected to electrophoresis in 7.5% polyacrylamide gels containing 0.1% SDS, electrotransferred onto nitrocellulose membranes (BioBlot; Costar Corp., Cambridge, MA), and blocked for 2 h with PBS containing 3% BSA at room temperature. After blocking, membranes were incubated with 0.1 μg/ml first antibodies in PBS containing 1% BSA, washed in PBS containing 0.1% Tween 20 (PBS-T), incubated with horseradish peroxidase–conjugated sheep anti–mouse IgG Ab (10,000 times dilution) in PBS-T, washed in PBS-T, and analyzed by the enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL). In the case of ^{125}I-labeled antibodies, membranes were incubated overnight in the cold room with ^{125}I-labeled antiphosphotyrosine mAb (4G10, kindly provided by Brian Druker, Dana-Farber Cancer Institute, Boston, MA) or 10G2 mAb in PBS-T, were washed in PBS-T, and were subjected to autoradiography. The method used in ^{125}I-labeling of antibodies was described elsewhere (11). Membranes were reprobed with other antibodies after reblocking. For overlay assay, GST-FAK fusion protein containing FAK residues 706–1052 was ^{125}I labeled by the same method and used instead of radiolabeled antibodies.

Microinjection and Immunofluorescence. GST-FAK fusion proteins were washed on beads with PBS, eluted from beads by the elution solution (20 mM glutathione, 50 mM Tris-HCl, final pH 8.0), and dialyzed with DMEM. Microinjection was performed based on the syringe-loading method described by Clarke and McNeil (27). Swiss 3T3 cells were trypsinized, collected by centrifugation, resuspended in 200–300 μl DMEM containing 2 mg/ml GST-FAK fusion proteins and 0.5% wt/vol Pluronic F-68 (Sigma Chemical Co.). Cells were passed through hypodermic needles (30G; Hamilton Co., Reno, NV) 30 times, collected by

centrifugation, and incubated overnight on a cover slip in 5% FCS-supplemented DMEM. For immunostaining, cells were fixed and immersed with PBS containing 3.7% formaldehyde and PBS containing 0.2% Triton X-100. Cells were then incubated with first antibodies in PBS (antipaxillin mAb, 1:50, or anti-GST mAb, 1:100), washed with PBS, incubated with FITC-conjugated goat anti-mouse IgG Ab (Tago, Inc., Burlingame, CA) in PBS (1:50), washed, and subjected to analysis with fluoromicroscopy (Axioskop; Carl Zeiss, Inc., Thornwood, NY).

Results

Identification of a Tyrosine-phosphorylated 70-kD pp125^{FAK}-associated Protein as Paxillin. It is now established that one of the major substrates for integrin-mediated tyrosine phosphorylation is pp125^{FAK}, a 125-kD cytoplasmic PTK. Furthermore, our recent studies indicated that the solid-phase cross-linking of β1 integrin by antibodies or its ligand FN stimulated tyrosine phosphorylation of several additional proteins, such as 140, 110–130, 70, 55–60, and 45 kD in peripheral T cells (27a) and in a T lymphoblastoid cell line, HPB-ALL (data not shown). To determine the relationship between pp125^{FAK} and these other proteins, we attempted to define pp125^{FAK}-associated molecules. For this purpose, lysates of HPB-ALL cells with or without FN stimulation were precipitated with the anti-FAK mAb (10G2) or with the GST fusion protein containing pp125^{FAK} COOH-terminal region residues 706–1052, and were analyzed by immunoblotting with antiphosphotyrosine mAb. As shown in Fig. 1, tyrosine-phosphorylated pp125^{FAK} was well precipitated with 10G2 mAb, whereas no additional tyrosine-phosphorylated proteins were detected by precipitation with 10G2 mAb (lane *6*). However, a 70-kD protein, which was precipitated by the GST-FAK fusion protein from the lysate of FN-stimulated HPB-ALL cells, was clearly detected by immunoblotting with antiphosphotyrosine mAb (Fig. 1 *A*, lane *8*). This 70-kD protein was not detected by antiphosphotyrosine mAb blotting in the absence of FN stimulation (Fig. 1 *A*, lane *4*). These results suggest the following: (*a*) a 70-kD protein is tyrosine phosphorylated by FN stimulation in HPB-ALL cells; (*b*) this 70-kD protein (pp70) binds to FAK protein, although it is not clear whether tyrosine phosphorylation of this protein is required for binding to pp125^{FAK}. Tyrosine phosphorylation in the binding site of pp125^{FAK} is not necessary for pp70 binding, because the GST-FAK fusion protein, which was not phosphorylated on tyrosine residues, bound to pp70. This tyrosine-phosphorylated 70-kD protein was also detected in H9 cells and Jurkat cells as well as peripheral T cells by the ligation of β1 integrins (data not shown). In addition to lymphoid cells, pp70 was also detected in nonlymphoid adherent cell lines such as T-47D, a human

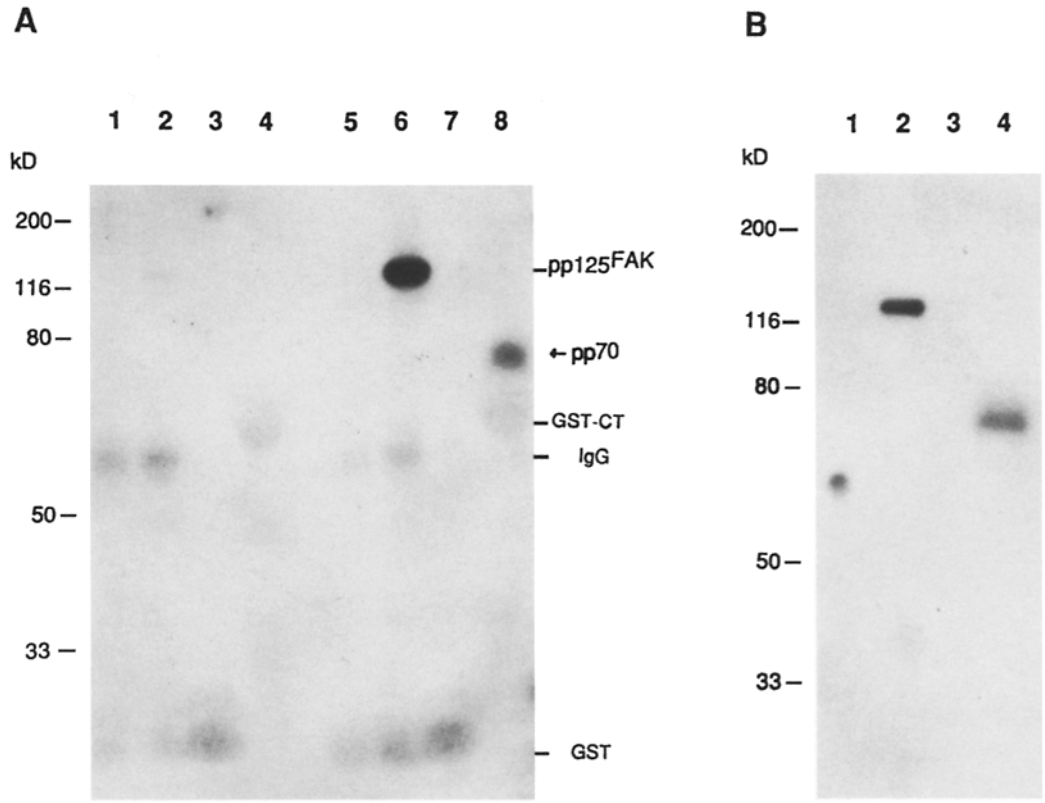

Figure 1. Identification of a tyrosine-phosphorylated 70-kD pp125^{FAK}-associated protein. (*A*) HPB-ALL cells were incubated in uncoated (lanes *1–4*) and FN-coated plates (lanes *5–8*). Cellular lysates were precipitated without first Ab (lanes *1* and *5*), with 10G2 mAb (lanes *2* and *6*), with GST protein (lanes *3* and *7*), and with GST-FAK fusion protein (lanes *4* and *8*) and analyzed by immunoblotting with antiphosphotyrosine mAb (4G10) and the ECL system. (*B*) T-47D cell lysates were analyzed by the same methods as *A* using ^{125}I-labeled 4G10 as a probe. Lanes *1–4* in *B* correspond to lanes *1–4* in *A*.

breast cancer cell line (Fig. 1 *B*, lane *4*). Given that preincubation of the GST-FAK fusion protein with the 10G2 mAb essentially blocked precipitation of pp70 (data not shown), it was conceivable that 10G2 mAb competed with pp70 for binding to pp125^{FAK}.

To identify the pp70 protein, pp70 was precipitated with the GST-FAK fusion protein, fractionated by SDS-PAGE, and analyzed by immunoblotting with specific antibodies against candidate proteins for pp70. As shown in Fig. 2 *A*, we found that paxillin was precipitated with GST-FAK fusion protein from HPB-ALL cell lysates with or without FN stimulation, as well as from T-47D cell lysate, and it migrated with a mobility similar to pp70, suggesting that pp70 was identical to the tyrosine-phosphorylated paxillin. It was also determined that tyrosine phosphorylation of paxillin was induced by the ligation of β1 integrins in HPB-ALL cells (data not shown) and in peripheral T cells (27a). To confirm that pp70 was paxillin, pp70 was precipitated by the GST-FAK fusion protein from T-47D cell lysate, solubilized by boiling in buffer containing 1% SDS, reprecipitated with antiphosphotyrosine mAb or antipaxillin mAb, and analyzed by immunoblotting with antipaxillin mAb (Fig. 2 *B*) and with antiphosphotyrosine mAb (Fig. 2 *C*). Tyrosine-phosphorylated pp70, which was precipitated with the GST-FAK fusion protein, was detected by antipaxillin mAb (Fig. 2 *B*, lane *4*), and tyrosine-phosphorylated paxillin (Fig. 2 *C*, lanes *1* and *5*) migrated with exactly the same mobility as pp70 (Fig. 2 *C*, lanes *2* and *4*). Similar results were obtained in FN-stimulated HPB-ALL cells (data not shown). These results confirm that pp70 is paxillin. Comparison of Fig. 2, *B* and *C*, (lanes *2* and *5*) showed that non–tyrosine-phosphorylated paxillin was precipitated with the GST-FAK fusion protein as well as pp70, tyrosine-phosphorylated paxillin. Furthermore, the paxillin protein that was precipitated with the GST-FAK fusion protein from HPB-ALL cell lysate without FN stimulation was not tyrosine phosphorylated (Fig. 1 *A*, lane *4*, and Fig. 2 *A*, lane *2*). These results strongly suggest that tyrosine phosphorylation of paxillin is not required for the binding to pp125^{FAK}.

Direct Association of pp125^{FAK} with Paxillin. Although the association of the GST-FAK fusion protein with paxillin was demonstrated, the following points remained unclear: (*a*) Is endogenous pp125^{FAK} associated with paxillin? (*b*) Is paxillin directly associated with pp125^{FAK} or associated indirectly via binding to other proteins? To define whether endogenous pp125^{FAK} protein was associated with paxillin, HPB-ALL and T-47D cell lysates were immunoprecipitated with antipaxillin mAb and analyzed by immunoblotting. As shown in Fig. 3, *A* and *B*, pp125^{FAK} was coprecipitated with paxillin by antipaxillin mAb (lanes *1–3*). The amounts of coprecipitated pp125^{FAK} appear to be almost the same with or without FN stimulation (Fig. 3 *A*, lanes *1* and *2*). Although paxillin was not coprecipitated with pp125^{FAK} by 10G2 mAb (Fig. 3 *B*, lane *4*), it should be noted that paxillin was coprecipitated with another anti-FAK mAb (Transduction Laboratories, Lexington, KY), which recognized a different epitope of FAK from 10G2 mAb (data not shown). These data further suggest that 10G2 mAb competes with paxillin for binding to pp125^{FAK}. pp125^{FAK} was also coprecipitated with crk, an adapter protein composed of SH2 and SH3 domains (28), using anti-crk mAb in T-47D cells (Fig. 3 *A*, lane *5*).

To determine whether the association between pp125^{FAK} and paxillin was direct or indirect, we next performed overlay assay using ^{125}I-labeled GST-FAK fusion protein as a probe. Immunoprecipitated paxillin was analyzed by overlay assay with ^{125}I-labeled GST-FAK fusion protein. As shown in Fig. 4 *A*, a protein of 68 kD was detected by ^{125}I-labeled GST-FAK fusion protein on the lanes of precipitates with antipaxillin mAb (lanes *1* and *2*), whereas no protein was detected using control precipitations (lanes *3* and *4*). This protein had the same mobility as paxillin by reprobing of the membrane with anti-paxillin mAb (Fig. 4

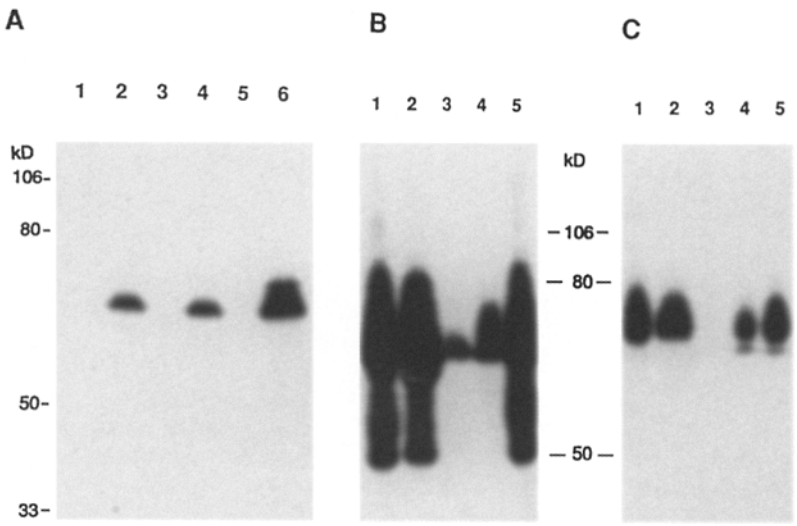

Figure 2. Identification of pp70 as paxillin. (*A*) Cellular lysates of HPB-ALL cells incubated in uncoated (lanes *1* and *2*) and FN-coated plates (lanes *3* and *4*) and T-47D cells (lanes *5* and *6*) were precipitated with GST protein (lanes *1*, *3*, and *5*) or GST-FAK fusion protein (lanes *2*, *4*, and *6*) and analyzed by immunoblotting with antipaxillin mAb (Transduction Laboratories) and the ECL system. (*B*) T-47D cell lysates were precipitated with antipaxillin mAb (lane *1*) or with GST-FAK fusion protein (lane *2*). After precipitation with GST-FAK fusion protein, associated molecules were solubilized and reprecipitated without first Ab (lane *3*), antiphosphotyrosine mAb (4G10)–conjugated beads (lane *4*), or antipaxillin mAb (lane *5*). Precipitates were analyzed by immunoblotting with antipaxillin mAb and the ECL system. Since five times more protein was used for precipitation than in *A*, nonspecific binding of paxillin to beads was observed in lane *3*. (*C*) The same membrane used in *B* was reprobed with ^{125}I-labeled antiphosphotyrosine mAb (4G10).

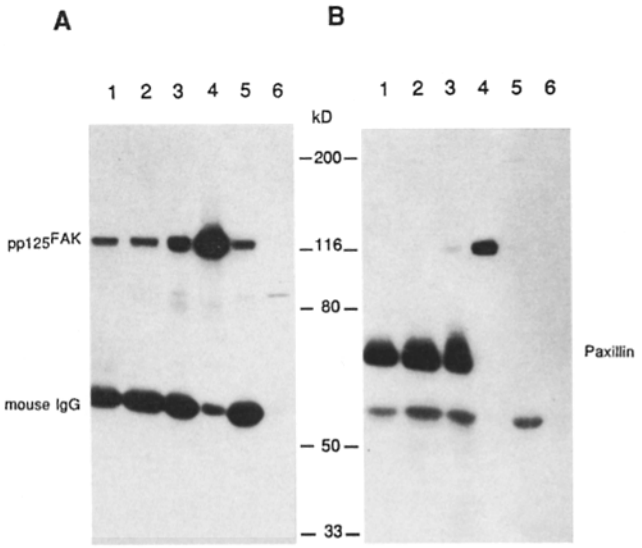

Figure 3. Coprecipitation of pp125^{FAK} with paxillin. (*A*) Cellular lysates of HPB-ALL cells incubated in uncoated (lane *1*) or FN-coated plate (lane *2*) and T-47D cells (lanes *3–6*) were immunoprecipitated with antipaxillin mAb (lanes *1–3*), 10G2 mAb (lane *4*), anti-crk mAb (Transduction Laboratories) (lane *5*), and without first Ab (lane *6*). Precipitates were analyzed by immunoblotting with anti-FAK mAb (Transduction Laboratories) and the ECL system. mAbs used for immunoprecipitation were detected at 55 kD. (*B*) The same membrane used in *A* was reprobed with antipaxillin Ab and the ECL system. A 125-kD protein in lanes *3* and *4* is pp125^{FAK} because of the remaining mAb of *A*.

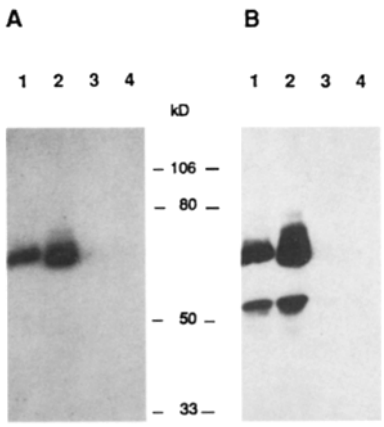

Figure 4. Direct association of paxillin with pp125^{FAK}. (*A*) Cellular lysates of HPB-ALL cells (lanes *1* and *3*) and T-47D cells (lanes *2* and *4*) were precipitated with antipaxillin mAb (lanes *1* and *2*) or without first Ab (lanes *3* and *4*). Precipitates were analyzed by overlay assay with ^{125}I-labeled GST-FAK fusion protein containing FAK residues 706–1052 and autoradiographed for 2 d. (*B*) The same membrane used in *A* was reprobed with antipaxillin mAb and the ECL system.

B). The above results demonstrate a direct association between pp125^{FAK} and paxillin.

Identification of Paxillin-binding Domain of pp125^{FAK}. To determine the paxillin-binding domain of pp125^{FAK}, we developed several deletion mutants derived from the GST-FAK fusion protein. HPB-ALL cell lysates were incubated with these deletion mutant proteins, precipitated, and analyzed by immunoblotting with antipaxillin mAb. As shown in Fig. 5 *A*, GST fusion protein containing pp125^{FAK} amino acid residues 706–1052 and 903–1052 retained paxillin-binding activity, whereas fusion proteins containing FAK residues 706–997 and 903–997 did not bind to paxillin. These results indicate that the paxillin-binding domain is located in pp125^{FAK} residues 903–1052. To define the paxillin-binding domain of pp125^{FAK} more precisely, we developed additional deletion mutants of the GST-FAK fusion protein by PCR and performed the paxillin-binding assay with these mutants. As shown in Fig. 5 *C*, deletion mutants containing pp125^{FAK} residues 896–1047, 896–1052, 903–1052, and 919–1052 precipitated paxillin, while mutants containing FAK residues 896–1038, 939–1052, 903–997, 896–1005, 896–1015, 896–1027, and 967–1052 did not precipitate paxillin. The amounts of the mutant proteins used for precipitation are shown in Fig. 5 *E*. From precipitation analysis with deletion mutants shown in Fig. 5 and the additional mutants (summarized in Table 1), we conclude that pp125^{FAK} residues 919–1042 are sufficient for the association with paxillin. Deletion mutants containing FAK residues 923–1052 or 896–1039 did not precipitate paxillin, suggesting that the amino acid residues of pp125^{FAK}, which are critical for paxillin binding, are located close to both ends of the paxillin-binding domain, residues 919–1042. This paxillin-binding domain of human pp125^{FAK} overlaps with the FAT domain of chick pp125^{FAK}. Hildebrand et al. reported that chick pp125^{FAK}

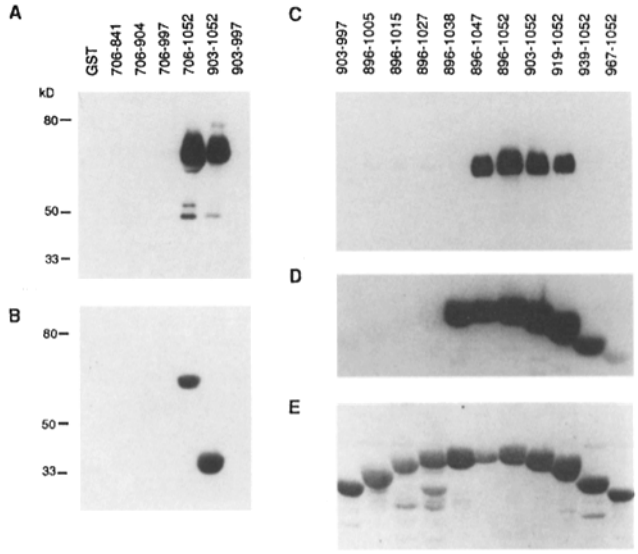

Figure 5. Paxillin binding analysis with deletion mutants of GST-FAK fusion proteins. (*A*) HPB-ALL cell lysates were precipitated with GST-FAK fusion proteins. Precipitates were analyzed by immunoblotting with antipaxillin mAb and ECL system. (*B*) The same membrane used in *A* was reprobed with ^{125}I-labeled 10G2 mAb. (*C*) HPB-ALL cell lysates were precipitated with deletion mutants of GST-FAK fusion proteins and analyzed by immunoblotting with antipaxillin mAb and the ECL system. (*D*) The same membrane used in *C* was reprobed with ^{125}I-labeled 10G2 mAb. (*E*) The same membrane used in *C* and *D* was stained with Ponceau S (Sigma Chemical Co.).

Table 1. *Summary of the Functions of GST-FAK Fusion Proteins*

Deletion mutants	10G2*	PB‡	FAT§	Substitution mutants		10G2	PB	FAT
706-1052	+	+	+	923	K → E	+	+	+
706-841	−	−	ND‖	928	V → G	±	−	−
706-904	−	−	−	929	T → A	+	+	ND
706-997	−	−	ND	929	T → S	+	+	ND
706-1038	+	−	−	931	L → R	±	−	−
896-1005	−	−	ND	933	K → E	+	+	ND
896-1015	−	−	ND	935	V → A	+	−	ND
896-1027	−	−	ND	1033	N → D	+	+	ND
896-1038	+	−	−	1034	L → S	+	−	−
896-1039	+	−	−	1035	L → A	+	−	ND
896-1042	+	+	ND	1036	D → H	+	+	+
896-1047	+	+	ND	1037	V → D	+	−	ND
896-1052	+	+	+	1039	D → A	+	−	ND
903-1052	+	+	+	1040	Q → E	+	+	ND
903-997	−	−	ND	1040	Q → G	+	+	ND
919-1052	+	+	+	1040	Q → K	+	+	ND
923-1052	+	−	ND	1042	R → G	+	−	ND
928-1052	+	−	−	1043	L → R	+	+	+
939-1052	+	−	−					
967-1052	±	−	ND					

Paxillin-binding activity, focal adhesion–targeting activity, and 10G2 mAb–binding activity of each GST-FAK fusion protein are summarized. Paxillin-binding activity represents the activity to precipitate paxillin from cellular lysates. Focal adhesion–targeting activity was determined by immunohistochemical analysis of the microinjected fusion protein. 10G2 mAb–binding activity was determined by immunoblotting with ^{125}I-labeled 10G2 mAb.

*10G2 mAb–binding activity.

‡ Paxillin-binding activity.

§ Focal adhesion–targeting activity.

‖ Not determined.

mutants with a deletion that corresponds to human pp125^{FAK} residues 861–967 or 969–1016 resulted in the loss of FAT (21). Since these two mutants have deletions in the paxillin-binding domain, these mutants are expected to lack paxillin-binding activity, suggesting the relevance between paxillin-binding activity and FAT of pp125^{FAK}. To determine the 10G2 mAb epitope, the same membranes used in Fig. 5, *A* and *C*, were reprobed with ^{125}I-labeled 10G2 mAb. As shown in Fig. 5, *B* and *D*, the epitope of 10G2 mAb was mapped within pp125^{FAK} residues 939–1038, indicating that the epitope of 10G2 mAb is located within the paxillin-binding domain of pp125^{FAK}.

Identification of pp125^{FAK} Amino Acid Residues Essential for Paxillin Binding. Paxillin was first identified as a vinculin-binding protein (23). Vinculin, a 116-kD cytoskeleton protein, is also localized at focal adhesions, and the paxillin-binding domain of vinculin was identified recently (24). Chick vinculin residues 881–1000 are sufficient for paxillin binding, and vinculin residues 979–1000 are critical for paxillin binding. Comparison of paxillin-binding domains between pp125^{FAK} and vinculin revealed several conserved amino acid residues. As shown in Fig. 6, two subdomains located on both ends of the paxillin-binding domain of pp125^{FAK} have homology with the paxillin-binding domain of vinculin. pp125^{FAK} residues 919–935 and vinculin residues 952–968 share a sequence, R/K X6 E/D X V T X L X3 V/L (paxillin-binding subdomain 1 [PBS1]). pp125^{FAK} residues 1034–1039 and vinculin residues 981–986 also share a sequence, L L X V X D/E (paxillin-binding subdomain 2 [PBS2]). To determine the role of these homologous amino acid residues in the interaction with paxillin, we generated substitution mutants within pp125^{FAK} residues 896–1052 and performed paxillin-binding analysis with these mutant proteins. As shown in Fig. 7 *A*, substitutions of either residue 928 Val to Gly, 931 Leu to Arg, and 935 Val to Ala in PBS1 resulted in a significant decrease in paxillin-binding activity, whereas substitutions of 923 Lys to Glu, 929 Thr to Ala or Ser, and 933 Lys to Glu had no effect on paxillin binding. In PBS2, substitutions of 1034 Leu to Ser, 1035 Leu to Ala, 1037 Val to Asp, 1039 Glu to Ala, and 1042 Arg to Gly significantly decreased paxillin-binding activity, whereas 1033 Asn to Asp, 1036 Glu to

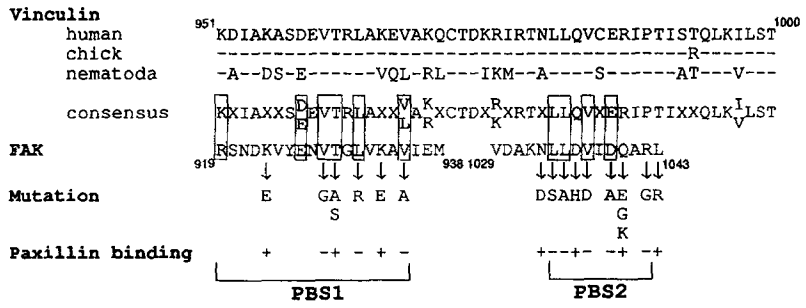

Figure 6. Sequence homology between pp125^{FAK} and vinculin. Amino acid sequences of the paxillin-binding domains of vinculin and pp125^{FAK}. Amino acid sequences of the paxillin-binding domains of human, mouse, and chick pp125^{FAK} are conserved. Conserved amino acids between pp125^{FAK} and vinculin are boxed. Substituted FAK residues used in the following analysis and paxillin-binding activity of each mutant are shown below FAK sequence.

His, 1040 Gln to Glu, Gly, or Lys, and 1043 Leu to Arg did not. These results clearly indicate that conserved or homologous amino acids between pp125^{FAK} and vinculin are essential for paxillin binding.

Biological Relevance between the Paxillin Binding and the FAT of pp125^{FAK}. Given that the paxillin-binding domain and the FAT domain of pp125^{FAK} overlap each other, one of the putative biological functions of pp125^{FAK} binding to paxillin would be the recruitment of pp125^{FAK} to focal adhesions. To elucidate this possibility, we performed immunohistochemical analysis combined with microinjection of GST-FAK fusion proteins. The GST-FAK fusion protein containing FAK residues 706–1052 (GST-FAK 706–1052) was microinjected into 3T3 Swiss albino cells by the syringe-loading method (27) and analyzed by immunostaining with anti-GST mAb and FITC-labeled anti-mouse IgG Ab. As shown in Fig. 8, *C* and *D*, microinjected GST-FAK 706–1052 was detected at focal adhesions and localized at the ends of actin stress fibers by double staining with rhodamine-labeled phalloidine (Fig. 8 *E*). Depending on the efficiency of microinjection (shown in Fig. 8 *B*), GST-FAK 706–1052 was not detected in all cells (Fig. 8 *C*), although endogenous paxillin was detected at focal adhesions in all cells (Fig. 8 *A*) as well as endogenous pp125^{FAK} and vinculin (data not shown). Microinjected GST protein, which did not contain a pp125^{FAK} peptide, was not detected at focal adhesions (Fig. 8 *F*). Next, the FAT of the deletion mutants of GST-FAK fusion proteins was determined by the same method. GST-FAK fusion proteins containing pp125^{FAK} residues 896–1052 (Fig. 8 *H*) and 919–1052 (Fig. 8 *J*), which had binding activity to paxillin, were localized to focal adhesions, whereas GST-FAK fusion proteins containing FAK residues 706–904 (Fig. 8 *G*), 896–1038 (Fig. 8 *I*), and 939–1052 (Fig. 8 *K*), which had no binding activity to paxillin, were not localized to focal adhesions. These results indicate that pp125^{FAK} residues 919–1052 are sufficient for the FAT of FAK. For further study of the role of paxillin binding in the FAT of pp125^{FAK}, similar analysis was performed using substitution mutants of GST-FAK 896–1052. GST-FAK mutants containing substitutions of 928 Gly (data not shown), 931 Arg (Fig. 8 *L*), and 1034 Ser (Fig. 8 *M*) were not localized to focal adhesions, whereas substitution mutants carrying 923 Glu (data not shown), 1036 His (Fig. 8 *N*), and 1043 Arg (data not shown) were localized to focal adhesions. These results, summarized in Table 1, clearly depict the relevance between paxillin-binding activity and FAT activity of pp125^{FAK}, indicating that pp125^{FAK} localizes to focal adhesions by its direct interaction with paxillin.

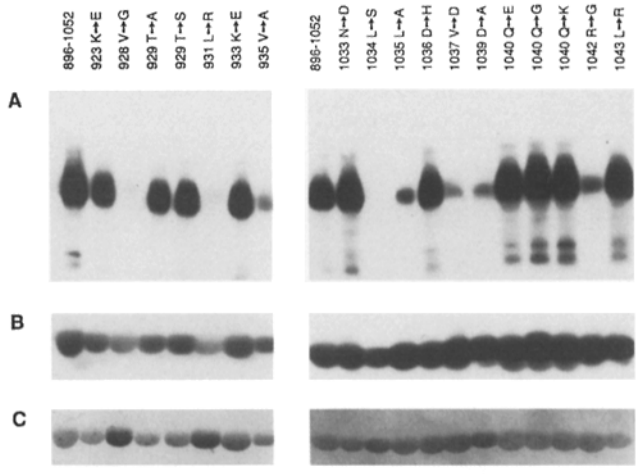

Figure 7. Paxillin binding analysis with substitution mutants of GST-FAK fusion proteins. (*A*) HPB-ALL cell lysates were precipitated with substitution mutants of GST-FAK fusion proteins and analyzed by immunoblotting with antipaxillin mAb and the ECL system. (*B*) The same membrane used in *A* was reprobed with ^{125}I-labeled 10G2 mAb. (*C*) The same membrane used in *A* and *B* was stained with Ponceau S.

Discussion

pp125^{FAK} colocalizes with β1 integrins at focal adhesions, where cells attach to the extracellular matrix (15, 19). The complex architecture of cellular focal adhesions depends on numerous protein–protein interactions between focal adhesion components. However, the mechanisms and cellular components regulating various aspects of signaling induced by integrins are unclear.

Our previous results indicated that the ligation of β1 integrins induces tyrosine phosphorylations of 140-, 110–130-, 70-, 55–60-, and 45-kD proteins. Some of these proteins have been identified to be phospholipase C γ, pp125^{FAK}, paxillin, pp59^{fyn}, and pp56^{lck}, and MAP kinase in peripheral T cells (27a). Since β1 integrins lack an intrinsic PTK activity, these protein tyrosine phosphorylations strongly suggest the presence of a PTK(s), which is functionally linked to β1 integrins. pp125^{FAK} is a prime candidate for such PTKs, since FAK is colocalized with β1 integrins at focal adhesions (15). pp125^{FAK} is also tyrosine phosphory-

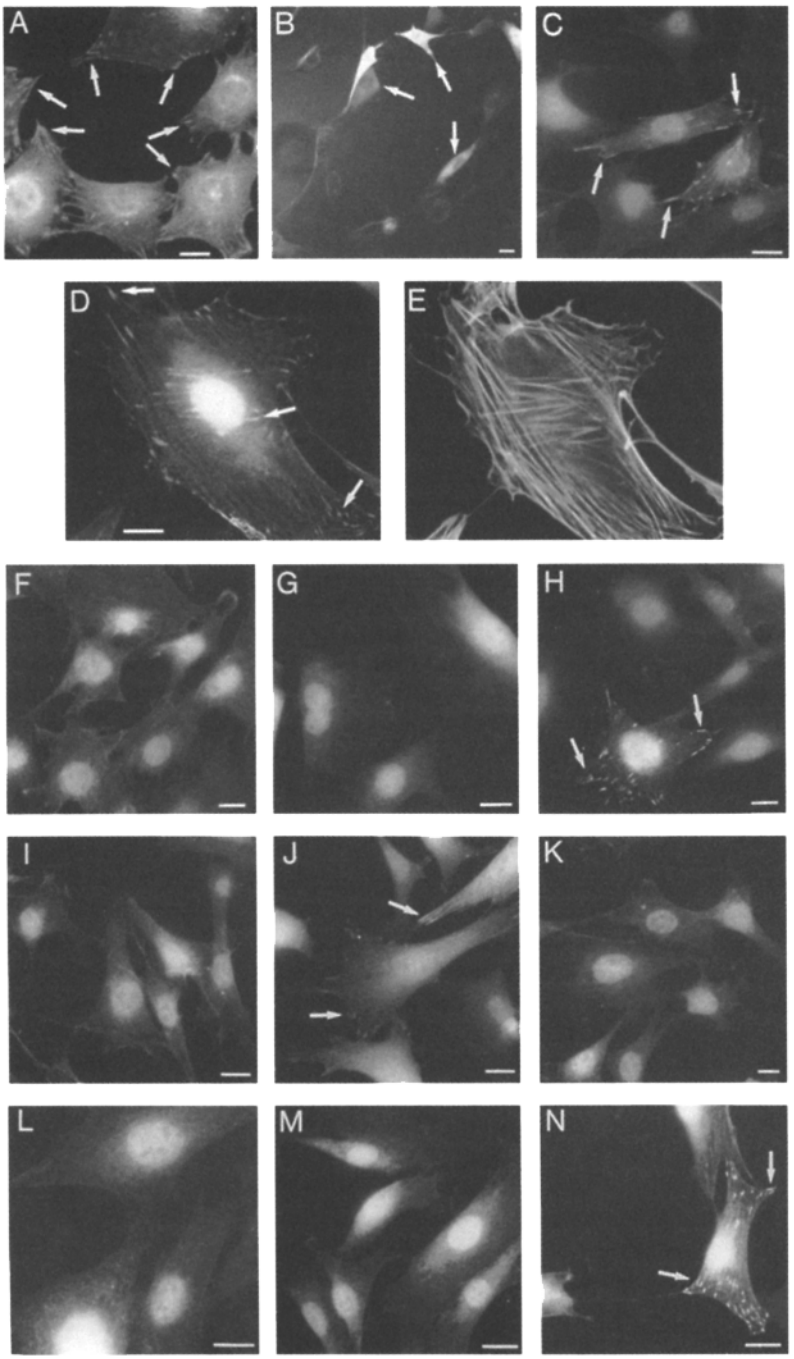

Figure 8. Immunohistochemical analysis of microinjected GST-FAK fusion proteins with anti-GST mAb in Swiss 3T3 cells. (*A*) Antipaxillin mAb staining of Swiss 3T3 cells with FITC-labeled goat anti–mouse IgG Ab (FITC-GαMIgG). Paxillin (*arrows*) is homogeneously stained at focal adhesions. (*B*) Live staining of FITC-dextran–injected Swiss 3T3 cells as a microinjection control. Swiss 3T3 cells were microinjected 10 mg/ml FITC-dextran (average MW 40 kD; Sigma Chemical Co.) by the syringe loading method. Cells were incubated on a cover slip and analyzed by fluoromicroscopy without fixation. Microinjected FITC-dextran was detected (*arrows*). (*C* and *D*) Anti-GST mAb staining of Swiss 3T3 cells that were microinjected with GST-FAK fusion protein containing FAK residues 706–1052. Swiss 3T3 cells microinjected GST-FAK fusion protein by the syringe loading method were fixed, stained with anti-GST mAb (Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-GαMIgG, and analyzed by fluoromicroscopy. Microinjected GST-FAK fusion proteins (*arrows*) were detected at focal adhesions. Because of the relatively high concentration of FITC-GaMIgG, the nucleus was nonspecifically stained. (*E*) rhodamine-labeled phalloidine (Sigma Chemical Co.) staining of the same cell as *D*. Actin stress fibers are stained. Compared with *D*, stainings in *D* are localized at the ends of actin stress fibers. (*F*) Anti-GST mAb staining of Swiss 3T3 cells that were microinjected with GST protein. Microinjected GST protein was not detected at specific sites including focal adhesions. (*G–N*) Anti-GST mAb staining of Swiss 3T3 cells that were microinjected GST-FAK fusion proteins. Microinjected fusion proteins contained FAK residues 706–904 (*G*), 896–1052 (*H*), 896–1038 (*I*), 919–1052 (*J*), and 939–1052 (*K*). Microinjected substitution mutants of FAK-GST fusion protein contained substitutions of 931 Leu to Arg (*L*), 1034 Leu to Ser (*M*), and 1036 Glu to His (*N*). Microinjected GST-FAK fusion proteins localized at focal adhesions are shown by arrows. Bars, 10 μm.

lated by the ligation of β1 integrins (18, 19), and tyrosine-phosphorylated pp125^{FAK} is an adaptor of signaling molecules carrying SH2 domains. These signaling molecules include pp59^{fyn} and pp60$^{v\text{-}src}$ (29, 30), csk (31), phosphatidylinositol 3-kinase (32), GRB2 (33), and crk (34). Recruitment of these signaling molecules to pp125^{FAK} appears to induce downstream signals. For example, activation of MAP kinase by the Ras pathway is reported to be induced by integrin–ligand binding (27a, 33, 35). These signals subsequently result in various biological effects induced by the ligation of β1 integrins. However, it is still unclear how pp125^{FAK} is linked to integrins and how FAK is tyrosine phosphorylated by the ligation of β1 integrins. In this report, we have shown the direct association of pp125^{FAK} with paxillin, and we have demonstrated a perfect correlation between the paxillin-binding ability of pp125^{FAK} and FAT of pp125^{FAK}.

Paxillin was first identified as a 68-kD vinculin-binding protein localized to focal adhesions (23). Paxillin is phosphorylated on both serine/threonine and tyrosine residues (19). Paxillin has four LIM domains in its COOH-terminal region that are flanked by a unique NH_2-terminal region, and

it lacks SH2 and SH3 domains (22). Tyrosine phosphorylation of paxillin is invariably accompanied by the tyrosine phosphorylation of pp125^{FAK}, suggesting a close relationship between pp125^{FAK} and paxillin (19, 36). Turner and Miller recently showed that pp125^{FAK} was precipitated with the GST-paxillin fusion protein (22), although it was not clarified whether pp125^{FAK} was directly associated with paxillin and whether pp125^{FAK} was localized to focal adhesions by the association with paxillin. We have shown that paxillin is directly associated with GST fusion protein containing pp125^{FAK} residues 706–1052 by peptide-to-peptide interaction and that posttranslational modifications are not involved in pp125^{FAK}–paxillin association. Endogenous pp125^{FAK} is also coprecipitated with paxillin, indicating the in vivo association between pp125^{FAK} and paxillin. The engagement of integrins and the subsequent tyrosine phosphorylation of pp125^{FAK} and paxillin appear to have no effect on this association, since the amount of pp125^{FAK} coprecipitated with paxillin and the amount of pp125^{FAK} in the insoluble fraction were not altered by the ligation of β1 integrins in HPB-ALL cells (Fig. 2 *A* and our unpublished data). The pp125^{FAK} residues 919–1042 were determined to be sufficient for the association of pp125^{FAK} with paxillin. This paxillin-binding domain of pp125^{FAK} shares sequence similarity with the paxillin-binding domain of vinculin, and substitutions of conserved amino acids resulted in the loss of paxillin-binding activity. Most of the essential amino acids for paxillin binding are leucine and valine, which are amino acids with hydrophobic side chains and which are involved in the formation of tertiary structures and/or a hydrophobic pocket(s). pp125^{FAK} residue 1039 Glu has a carboxyl group, which is essential for paxillin binding, since the substitution of this glutamate residue to alanine resulted in a significant decrease of paxillin-binding activity. Despite lacking homology with vinculin sequence, 1042 Arg also appears to be essential for paxillin binding. These two paxillin domains of pp125^{FAK} and vinculin share homologous amino acids, although they also have several differences. In pp125^{FAK}, two paxillin-binding subdomains are separated by the insertion of 98–amino acid sequence, while PBS1 and -2 are closely located in vinculin. A putative binding site of PI3 kinase 85-kD subunit is located in this insertion sequence of pp125^{FAK} (37), and the GRB2-binding site of pp125^{FAK}, which included 925 Tyr, is located in the FAK PBS1 (33). These two binding sites for PI3 kinase and GRB2 are not located in vinculin PBS1, suggesting a difference between pp125^{FAK} and vinculin in downstream signalings.

The biological relevance between the paxillin-binding activity and the FAT activity of pp125^{FAK} was clearly demonstrated by the immunohistochemical analysis of the cells microinjected with GST-FAK fusion proteins. Deletion and substitution mutants with paxillin-binding activity are localized to focal adhesions, whereas mutants without paxillin-binding activity are not localized to focal adhesions. These findings indicate that pp125^{FAK} is localized to focal adhesions by the association with paxillin. Focal adhesions, where cells attach to substrata via integrin–ECM binding, are also composed of cytoskeletal proteins such as talin, vinculin, paxillin, FRNK, and pp125^{FAK} (19, 38). Among these proteins, vinculin, which has a conserved paxillin-binding domain with pp125^{FAK}, appears to be localized to focal adhesions by the association with paxillin (24), and talin is associated with vinculin (39). Our findings strongly suggest that pp125^{FAK} and other proteins are localized to focal adhesions and linked to β1 integrins via the interaction with paxillin. It remains to be clarified how pp125^{FAK} and paxillin are tyrosine phosphorylated by the ligation of β1 integrins. The interaction with paxillin appears not to be sufficient for tyrosine phosphorylation of pp125^{FAK}, since non–tyrosine-phosphorylated pp125^{FAK} is associated with paxillin in HPB-ALL cells without the ligation of β1 integrins. On the other hand, the induction of pp125^{FAK} tyrosine phosphorylation is much stronger by the solid-phase cross-linking of β1 integrins on plates than by the cross-linking in liquid phase (our unpublished data). This finding suggests that focal adhesion formation and FAT of pp125^{FAK} via paxillin play a crucial role in β1 integrin–mediated tyrosine phosphorylation of pp125^{FAK}.

In summary, pp125^{FAK} is associated with paxillin and localized to focal adhesions via paxillin. β1 integrin–ligand ligation induces FAT, and tyrosine phosphorylation of pp125^{FAK} then induces recruitment of signaling molecules to tyrosine-phosphorylated pp125^{FAK}, which presumably results in β1 integrin–mediated cell adhesion, cytoskeleton organization, and cell proliferation.

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