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Paxillin, a Tyrosine Phosphorylated Focal Adhesion-associated Protein Binds to the Carboxyl Terminal Domain of Focal Adhesion Kinase

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Focal adhesion kinase ($pp125^{FAK}$ or FAK) and paxillin colocalize with integrins in structures called focal adhesions. $pp125^{FAK}$ plays an important role in the transmission of integrin-induced cytoplasmic signals. Paxillin has also been implicated in cell signaling by virtue of its association with the protein tyrosine kinases $pp60^{src}$ and Csk (C-terminal Src kinase) as well as with the adapter/oncoprotein $p47^{gag\text{-}crk}$. In this report we show that endogenous $pp125^{FAK}$ and paxillin form a stable complex both in vivo and in vitro and that this interaction is direct, requiring only $pp125^{FAK}$ and paxillin. The paxillin binding site on $pp125^{FAK}$ has been localized to the carboxy-terminal 148 residues of $pp125^{FAK}$, but appears to be distinct from the previously identified focal adhesion–targeting sequence also present in the carboxy-terminal domain of $pp125^{FAK}$. The interaction of paxillin and $pp125^{FAK}$ is independent of the adhesion of cells to the extracellular matrix, as the association can be detected in suspension cells as well as those attached to fibronectin.

INTRODUCTION

The integrin family of cell surface receptors plays critical roles in multiple biological functions (Hynes, 1992). The integrins are noncovalently associated heterodimers whose extracellular domains bind either components of the extracellular matrix (ECM)[1] or other cell surface receptors (Albelda and Buck, 1990), while the cytoplasmic domains bind specific components of the actin cytoskeleton. Thus integrins appear to provide a structural linkage between the extracellular environment and the cellular cytoplasm (reviewed in Burridge *et al.*, 1988; Turner and Burridge, 1991). Mounting evidence indicates that integrin-ECM interactions function beyond the role of cell/cytoskeletal anchorage because these interactions induce intracellular phenomena classically attributed to activation of signal cascades (reviewed in Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993). Integrin binding to ECM or cross-linking of cell surface integrin leads to a pronounced increase in the tyrosine phosphorylation of focal adhesion kinase $pp125^{FAK}$ (Guan *et al.*, 1991; Burridge *et al.*, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1993) and a concomitant increase in its in vitro catalytic activity (Guan and Shalloway, 1992; Lipfert *et al.*, 1992). Therefore, $pp125^{FAK}$ is a candidate for mediating signal transduction after integrin-ligand interactions.

Focal adhesion kinase is comprised of three domains, a central catalytic domain flanked by amino- and carboxy-terminal noncatalytic domains that lack Src-homology 2 (SH2) and Src-homology 3 (SH3) domains. We have previously demonstrated that a 159-amino acid domain (the focal adhesion–targeting sequence or FAT sequence) within the carboxy-terminus

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[1] Abbreviations used: CE, chicken embryo; cFAK, carboxy-terminal epitope-tagged focal adhesion kinase; dl, deletion; ECM, extracellular matrix; FAK or $pp125^{FAK}$, focal adhesion kinase; FAT, focal adhesion targeting; GST, glutathione-*S*-transferase; GuHCl, guanidine hydrochloride; HMK, heart muscle kinase; RIPA, radioimmunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SH2 domain, Src-homology 2 domain; SH3 domain, Src-homology 3 domain; Simian virus 40, SV40.

of pp125^{FAK} is required for proper subcellular localization to focal adhesions, presumably by mediating an interaction with another cytoskeletal protein (Hildebrand *et al.*, 1993). Furthermore, in vivo and in vitro experiments have demonstrated that pp125^{FAK} undergoes autophosphorylation, predominantly on a single tyrosine Tyr^{397} (Schaller *et al.*, 1994). Phosphorylation of Tyr^{397} creates a high affinity binding site for SH2 containing proteins, including pp60^{src} and p59^{fyn} (Cobb *et al.*, 1994; Schaller *et al.*, 1994), thus providing a mechanism for the recruitment of kinases and other SH2 domain-containing proteins to focal adhesions.

Paxillin is a 68,000–75,000 dalton focal adhesion protein originally identified in chicken embryo fibroblasts transformed by Rous sarcoma virus (Glenney and Zokas, 1989). In addition to transformation by Rous sarcoma virus, a number of extracellular stimuli induce paxillin tyrosine phosphorylation, including neuropeptides (Zackary *et al.*, 1993), lysophosphatidic acid (Seufferlein and Rozengurt, 1994), PDGF (Rankin and Rozengurt, 1994), and fibronectin (Burridge *et al.*, 1992). These data imply that paxillin may be involved in multiple biological processes, with tyrosine phosphorylation modulating its function or activity. Interestingly, many of these same stimuli induce pp125^{FAK} tyrosine phosphorylation as well (Kumagai *et al.*, 1993; Sinnett-Smith *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994). Paxillin has also been implicated in numerous protein-protein interactions, including associations with vinculin (Turner *et al.*, 1990), p47$^{gag\text{-}crk}$ (Birge *et al.*, 1993), Csk (Sabe *et al.*, 1994), and pp60^{src} (Weng *et al.*, 1993), however the biological ramifications of these interactions are yet to be defined.

We have initiated a search to identify cellular proteins that physically associate with focal adhesion kinase and may therefore play a role in integrin/pp125^{FAK} signaling. In this report we demonstrate that paxillin and pp125^{FAK} form a physical complex in vivo and this interaction can be reconstituted in vitro. The paxillin binding site resides in the carboxy-terminal domain of pp125^{FAK} and overlaps the FAT sequence, also located in the carboxy-terminus of pp125^{FAK}. Efforts to map the paxillin binding site have also functioned to more precisely define the boundaries of the FAT sequence, encompassing residues 904 through 1040. Although the paxillin binding sequence and the FAT sequence appear to extensively overlap, the two domains can be functionally separated, indicating that paxillin may not solely be responsible for mediating the subcellular localization of pp125^{FAK}. Furthermore, analysis of the paxillin-pp125^{FAK} interaction after cell adhesion to fibronectin indicates that the interaction is constitutive and not significantly regulated by engagement of integrins with ECM.

MATERIALS AND METHODS

Cells and Viruses

Chicken embryo (CE) cells were prepared as described (Reynolds *et al.*, 1989). cDNAs encoding pp125^{FAK} and pp125^{FAK} variants were introduced into CE cells using the replication competent retroviral expression vector RCAS A (BH) (Hughes *et al.*, 1987; Hildebrand *et al.*, 1993; Schaller *et al.*, 1993) and the cells were lysed in modified radioimmunoprecipitation assay buffer (RIPA) (Kanner *et al.*, 1989). For some assays, cells were removed from tissue culture dishes by trypsinization, washed in soybean trypsin inhibitor (0.5 mg/ml) three times, resuspended in serum-free media (DMEM), and subsequently plated on bacterial plastic dishes that had been coated with either poly-L-lysine (0.2 mg/ml) or fibronectin (5 $\mu g/cc^2$). Cells were allowed to adhere for given amounts of time and were lysed in modified RIPA buffer.

Immunoprecipitation and In Vitro Association

pp125^{FAK} and pp125^{cFAK} (a variant containing an epitope tag at the carboxy-terminus derived from Simian virus 40 [SV40] large T antigen, consisting of the amino acid sequence KPPTPPPEPET) (Hildebrand *et al.*, 1993, Schaller *et al.*, 1993) were immunoprecipitated from 500 μg CE lysate using either the rabbit polyclonal serum BC3 (Schaller *et al.*, 1992) or mAb 2A7 (Kanner *et al.*, 1990) as described. Immune complexes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and Western blotting using either a paxillin-specific mAb (Transduction Laboratories, Lexington, KY), the pp125^{FAK}-specific sera BC3 (Schaller *et al.*, 1992), the pp125^{FAK}-specific mAb 2A7, or the SV40 large T antigen-specific mAb KT3 (MacArthur and Walter, 1984). For in vitro binding experiments, CE lysates were precleared with glutathione-*S*-transferase (GST) bound to glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ.). After preclearing, approximately 1 μg GST-FAK fusion protein immobilized onto glutathione-Sepharose was added to 500 μg CE lysates and incubated for 1 h at 4°C with constant rocking. The beads were washed twice with modified RIPA and twice with Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl). Bound protein complexes were eluted in Laemmli sample buffer and analyzed by Western or "farwestern" blotting.

Mutagenesis of pp125^{FAK} and Preparation of GST-FAK Fusion Proteins

All pp125^{FAK} or GST-FAK deletion mutants are designated by the amino acid residues that are deleted in the variant as determined from the cDNA sequence of avian *FAK* (Schaller *et al.*, 1992). *FAK* cDNA sequences encoding amino acid residues 687-1054 were cloned into the bacterial expression vector pGex2TK (Kaelin *et al.*, 1992). The resulting construct pGex2TK-cterm encodes the fusion protein Cterm. Subsequently, five deletion mutations of pGex2TK-cterm were generated that encode Cterm variants Ctermdl721–857, Ctermdl853–963, Ctermdl965–1012, Ctermdl965–1035, and Ctermdl1011–1052. For pp125^{cFAK} and the Cterm variant Ctermctag, the distal 13 carboxy-terminal residues have been replaced by an 11-residue epitope tag. Deletion mutants dl879–903, dl929–953, dl954–973, dl974–998, and dl999–1023 were generated by site-directed mutagenesis in either the full length, wild-type *FAK* cDNA or the c*FAK* cDNA (encoding the epitope-tagged variant pp125^{cFAK}) using the Altered Sites mutagenesis system (Promega, Madison, WI). The oligonucleotides used were as follows: dl879–903; AAGAAGCCCCCTCGCAAGCCACAGGAAATC, dl929–953; TATGAGAATGTAACCAAGGAGGTTGGCTTG, dl954–973; TACGTGCCCATGGTACTTCCTGCAAGCACC, dl974–998; CAGTCGCTGCCAGTGAAGATGAAGCTGGCC, dl999–1023; GCTGAGCTCATTAACCACGCTCTGGCTGTG. For evaluation in vitro, the mutations were cloned into the context of pGex2TK-cterm. Bacterial expression, purification, and in vitro labeling of GST fusion proteins

was performed as described (Kaelin *et al.*, 1992). Construction of pp125^{FAK} amino terminal deletion variants dl31–144 and dl51–377 and carboxy-terminal deletion variants dl721–857, dl853–963, and dl965–1012, has been described previously (Hildebrand *et al.*, 1993).

"Farwestern" Blotting (Gel-Overlay)

CE cell lysate and protein complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Nitrocellulose filters were denatured/renatured at 4°C as follows. Filters were incubated twice for 5 min in 100 ml hyb75 (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% NP-40) (Kaelin *et al.*, 1992) plus 6 M guanidine hydrochloride (GuHCl). The filters were washed four times for 10 min per wash in 100 ml hyb75 containing 3 M GuHCl (a 1:1 dilution of the 6 M GuHCl solution). This step was repeated four times, each time using hyb75 containing a twofold dilution of the GuHCl from the previous wash step. Filters were transferred to new dishes and washed twice for 30 min in 100 ml hyb75. Filters were then blocked for 30 min in 10 ml hyb75 plus 5% milk, then 30 min in 10 ml hybridization solution plus 1% milk as described (Kaelin *et al.*, 1992). After the blocking step, ^{32}P-labeled fusion protein was added (3×10^5 cpm/ml, specific activity of approximately 10^8 cpm/μg) and the filters were incubated for 8 h at 4°C. Filters were washed three times in 100 ml hyb75 plus 1% milk for 15 min at room temperature and exposed to film at −70°C with an intensifying screen. Generation of ^{32}P-labeled GST-FAK probe was as follows. GST-Cterm (described above) was expressed in *Escherichia coli*, affinity purified using glutathione Sepharose, and phosphorylated in vitro using cAMP-dependent protein kinase catalytic subunit (PKA, Sigma Chemical, St. Louis, MO) as described (Kaelin *et al.*, 1992). Briefly, 3 mg of GST-Cterm coupled to beads was washed in heart muscle kinase (HMK) buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 12 mM $MgCl_2$), resuspended in 40 ml of HMK containing 1 mM dithiothreitol, 50 U PKA, and 100 μCi ^{32}P-γ-ATP (6000 Ci/mmol, Dupont NEN, Wilmington, DE), and incubated for 45 min at room temperature. Beads were subsequently washed once with 1 ml HMK stop buffer (10 mM sodium phosphate [pH 8.0], 10 mM sodium pyrophosphate, 10 mM EDTA) and five times with 1 ml Tris-buffered saline. GST fusion proteins were eluted twice at room temperature with constant rotation in 10 bed volumes of glutathione (20 mM glutathione, 100 mM Tris [pH 8.0], 120 mM NaCl).

Immunofluorescence

For determining the subcellular localization of pp125^{FAK} mutants, CE cells expressing pp125^{cFAK} deletion variants were grown overnight on coverslips, fixed with 3.7% paraformaldehyde, permeabilized with 0.4% Triton X-100, and stained by indirect immunofluorescence with the mAb KT3 as previously described (Reynolds *et al.*, 1989; Wu *et al.*, 1991, Hildebrand *et al.*, 1993).

RESULTS

Co-Precipitation of Paxillin and pp125^{FAK}

Previous experiments demonstrated that sequences within the carboxy-terminal noncatalytic domain of pp125^{FAK} were required for efficient localization to focal adhesions. To identify proteins that might physically complex with pp125^{FAK} and direct subcellular localization, CE cells were infected with a replication competent retroviral vector designed to express pp125^{FAK} (Hildebrand *et al.*, 1993; Schaller *et al.*, 1993). pp125^{FAK} immune complexes prepared from cell lysates of such cells were probed with specific antibodies to determine the presence of co-precipitating focal adhesion proteins. Immunoprecipitation of pp125^{FAK} with either the polyclonal antiserum BC3 or mAb 2A7 resulted in the coimmunoprecipitation of approximately 5–10% of the paxillin present in the cell lysate (Figure 1). The 44-kDa protein detected in the paxillin Western blots is suspected to be a proteolytic cleavage product of paxillin that contains both the binding site for pp125^{FAK} and the epitope for the paxillin-specific antibody. Paxillin was not immunoprecipitated by either preimmune rabbit serum or by rabbit anti-mouse IgG (Figure 1) nor was paxillin directly recognized by the anti-pp125^{FAK} antibodies by Western blotting. Similarly, paxillin immune complexes con-

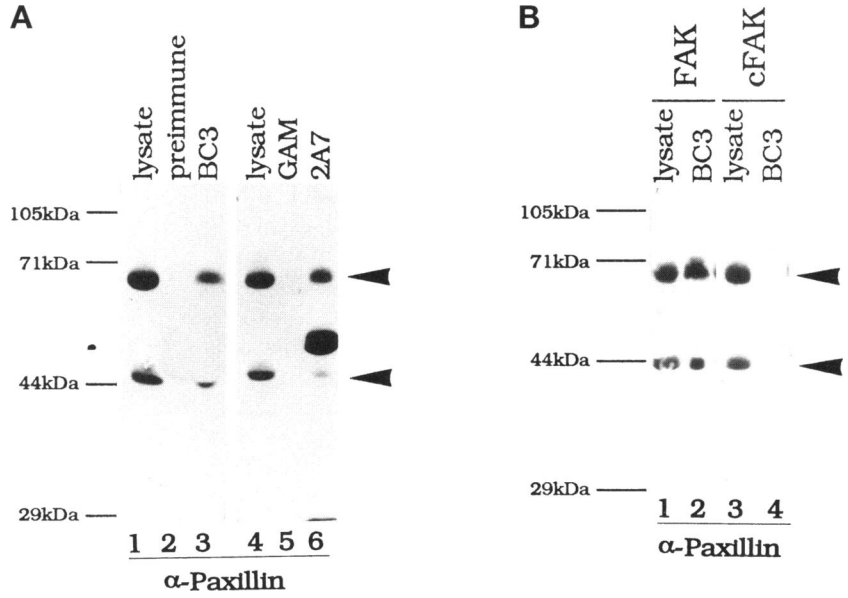

Figure 1. Coimmunoprecipitation of pp125^{FAK} and paxillin. cDNAs expressing either wild-type pp125^{FAK} or epitope-tagged variant pp125^{cFAK} were expressed in CE cells using the retroviral vector RCAS A (BH). pp125^{FAK} (panel A, lanes 1–6, and panel B, lanes 1 and 2) or pp125^{cFAK} (panel B, lanes 3 and 4) were immunoprecipitated from 500 μg CE cell lysate using the pp125^{FAK}-specific rabbit polyclonal sera BC3 or mAb 2A7 as indicated. Immune complexes were evaluated by Western blotting using a paxillin-specific mAb. To estimate binding efficiency, 50 μg of total CE lysate was loaded and evaluated directly (lysate). Preimmune and GAM (goat anti-mouse) designate nonspecific antibody controls. Arrowheads denote paxillin (68 kDa) and the presumed proteolytic fragment of paxillin (44 kDa).

tained pp125^{FAK} (our unpublished results). These initial experiments suggested an interaction between pp125^{FAK} and the cytoskeletal protein paxillin.

To further characterize the pp125^{FAK}-paxillin interaction, the location of the paxillin binding site within pp125^{FAK} was explored using a series of previously described pp125^{FAK} deletion variants (Hildebrand *et al.*, 1993). Immune complexes of wild-type pp125^{FAK} or pp125^{FAK} amino-terminal deletion variants dl31–144 and dl51–377 contained equal amounts of paxillin demonstrating that paxillin did not bind within the amino-terminal domain of pp125^{FAK} (our unpublished results). The carboxy-terminal deletion variant dl721–857 also stably associated with paxillin (our unpublished results). In contrast, a variant of pp125^{FAK}, pp125^{cFAK}, in which amino acids 1041–1054 were replaced with an 11-amino acid nonhomologous epitope tag failed to complex with paxillin (Figures 1 and 3A). This observation implicated the extreme carboxy-terminus of pp125^{FAK} in the interaction with paxillin. Delineation of a paxillin binding site using coimmunoprecipitation was complicated by our previous observation that deletions between residues 857 and 1012 abolish the ability of pp125^{FAK} to localize to cellular focal adhesions (Hildebrand *et al.*, 1993). Therefore, failure of various pp125^{FAK} carboxy-terminal mutations to complex with paxillin may not necessarily be due to alteration of the paxillin binding site, but actually may reflect a failure of pp125^{FAK} and paxillin to colocalize in the correct intracellular compartment.

The In Vivo Association of Paxillin and pp125^{FAK} Is Constitutive

To determine if the association of paxillin and pp125^{FAK} was regulated, stable association was evaluated under conditions during which new focal adhesions were being formed. Normal, untransfected CE cells were plated on dishes coated with either poly-L-lysine or fibronectin as described in MATERIALS AND METHODS. Cells were allowed to adhere and spread for the times indicated and were subsequently lysed. Endogenous pp125^{FAK} was immunoprecipitated using BC3 and the immune complexes were evaluated by Western blotting. As seen in Figure 2, pp125^{FAK} tyrosine phosphorylation increased as cells adhered and spread on fibronectin, but not as cells adhered to poly-L-lysine. Conversely, the amount of paxillin bound to pp125^{FAK} appeared to remain constant. These data indicate that the paxillin-pp125^{FAK} complex is stable, and not regulated in the same manner as pp125^{FAK} tyrosine phosphorylation. Under these conditions tyrosine phosphorylation of paxillin was not detectable.

Paxillin and pp125^{FAK} Associate In Vitro

The previous experiments indicated that the paxillin binding site resided within the carboxy-terminal noncatalytic domain of pp125^{FAK}, with the distal 13 residues being required for the interaction. To explore the association more thoroughly, we sought to establish this interaction in vitro using a GST fusion protein containing carboxy-terminal residues 687-1054 of pp125^{FAK} (Cterm) (Figure 3B). CE lysates were incubated with Cterm fusion protein immobilized on glutathione-Sepharose beads, the beads were washed, and bound proteins were subsequently analyzed by Western blotting using a paxillin-specific mAb antibody. As shown in Figure 4 (lane 3), paxillin present in the lysate formed a stable complex with the carboxy-terminus of pp125^{FAK}. Conversely, no paxillin association was observed using immobilized GST alone (lane 2), indicating that paxillin binding was mediated by pp125^{FAK}-specific sequences. By comparing the amount of paxillin detected by Western blotting in lane 1 with lanes 3 or 6, we estimate that approximately 10% of the paxillin in the CE lysates complexed with the fusion protein in vitro. Next, using a series of seven deletion variants of Cterm (Figure 3B), we attempted to elucidate the binding site for paxillin. Replacement of the final 13 residues of Cterm with unrelated sequences (Ctermctag) abolished paxillin binding (lane 4), thus confirming the co-precipitation experiments and implicating the extreme carboxy-terminal sequences of pp125^{FAK} in paxillin binding. Similarly, a truncated variant of Cterm, Ctermdl1011–1052, did not complex with paxillin (lane 5). Deletion of sequences 721–857 did not effect paxillin binding (lane 6), whereas several small deletions between residues 853 through 1035 abolished the ability of the carboxy-terminal domain to bind paxillin. These data indicated that either the binding site for paxillin resides within these sequences or these sequences are critical for maintaining the proper folding and/or conformation of the actual binding sequence.

Paxillin Binding to pp125^{FAK} Is Direct

The above experiments did not establish whether the paxillin-pp125^{FAK} interaction is direct or indirect. To evaluate these possibilities, we employed farwestern blot/gel overlay analysis using in vitro–labeled Cterm. The Cterm fusion protein was isolated, labeled with ^{32}P, and used to directly probe proteins immobilized on nitrocellulose membranes. As shown in Figure 5, labeled Cterm bound a single detectable protein in total cell lysate (Figure 5A, lane 1). Similarly, ^{32}P-labeled fusion protein bound to a 68-kDa protein that associates with Cterm in vitro (Figure 5A, lane 3). Conversely, the ^{32}P-labeled fusion protein did not bind to any proteins that associate with GST alone (Figure 5A, lane 2). To confirm that this protein was

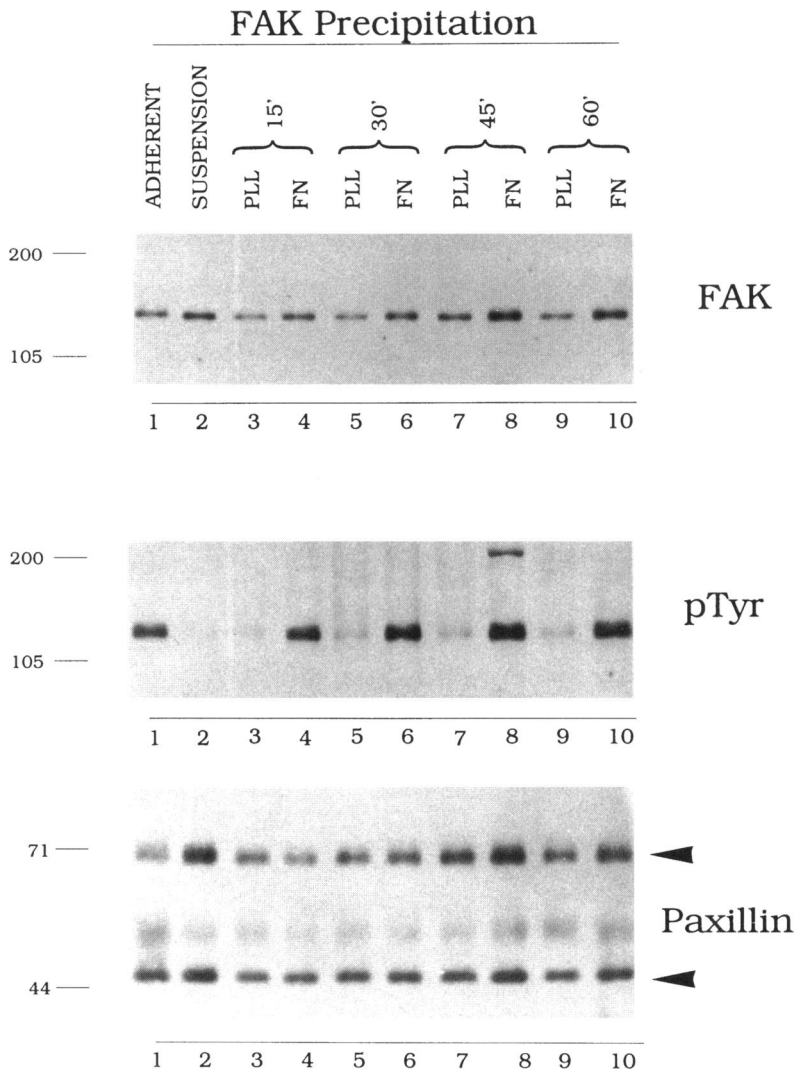

Figure 2. Regulation of pp125^{FAK}/paxillin complex formation by integrin-ECM interaction. CE cells were plated on fibronectin (FN) or poly-L-lysine for the times indicated as described in MATERIALS AND METHODS. pp125^{FAK} was immunoprecipitated using BC3, and the immune complexes were subsequently evaluated by Western blotting to detect pp125^{FAK} (FAK), phosphotyrosine (pTyr), and paxillin. Arrowheads denote paxillin and the presumed proteolytic fragment of paxillin.

paxillin, immune complexes were prepared from 1 mg of total cell lysate using a paxillin-specific mAb and the complexes were subjected to farwestern analysis (Figure 5B). ^{32}P-labeled Cterm bound to a 68-kDa protein that was immunoprecipitated by the anti-paxillin antibody (Figure 5B, lane 2). Upon longer exposure of the nitrocellulose membranes to film, the 44-kDa fragment is detected in farwestern blots of both total cell lysate and paxillin immune complexes (our unpublished observations). ^{32}P-labeled Ctermctag did not bind to immunoprecipitated paxillin (our unpublished results). These data indicate that the association between pp125^{FAK} and paxillin is direct and does not involve an intermediary protein.

Paxillin Binding Is Closely Linked to Subcellular Localization of pp125^{FAK}

The above data indicate that pp125^{FAK} carboxy-terminal residues 857-1054 are critical for the in vitro association of paxillin and pp125^{FAK}. These sequences extensively overlap the sequences required for the correct subcellular localization of pp125^{FAK} (Hildebrand *et al.*, 1993). However, the paxillin binding sequence and the FAT sequence can be functionally separated because replacement of the terminal 13 residues of pp125^{FAK} disrupts paxillin binding but does not effect localization (Hildebrand *et al.*, 1993; Schaller *et al.*, 1993). To further define the paxillin binding sequences and the FAT sequence, five nonoverlapping deletion mutations in the FAT sequence were generated by site-directed mutagenesis (Figure 3A). The resulting mutations were then assayed in vivo, in the context of pp125^{cFAK}, for an affect upon subcellular localization. Each of the five pp125^{cFAK} deletion variants were initially evaluated by immunoprecipitation and Western blotting (Figure 6). pp125^{cFAK} deletion variants were expressed in CE cells, immunoprecipitated from 500 μg total cell

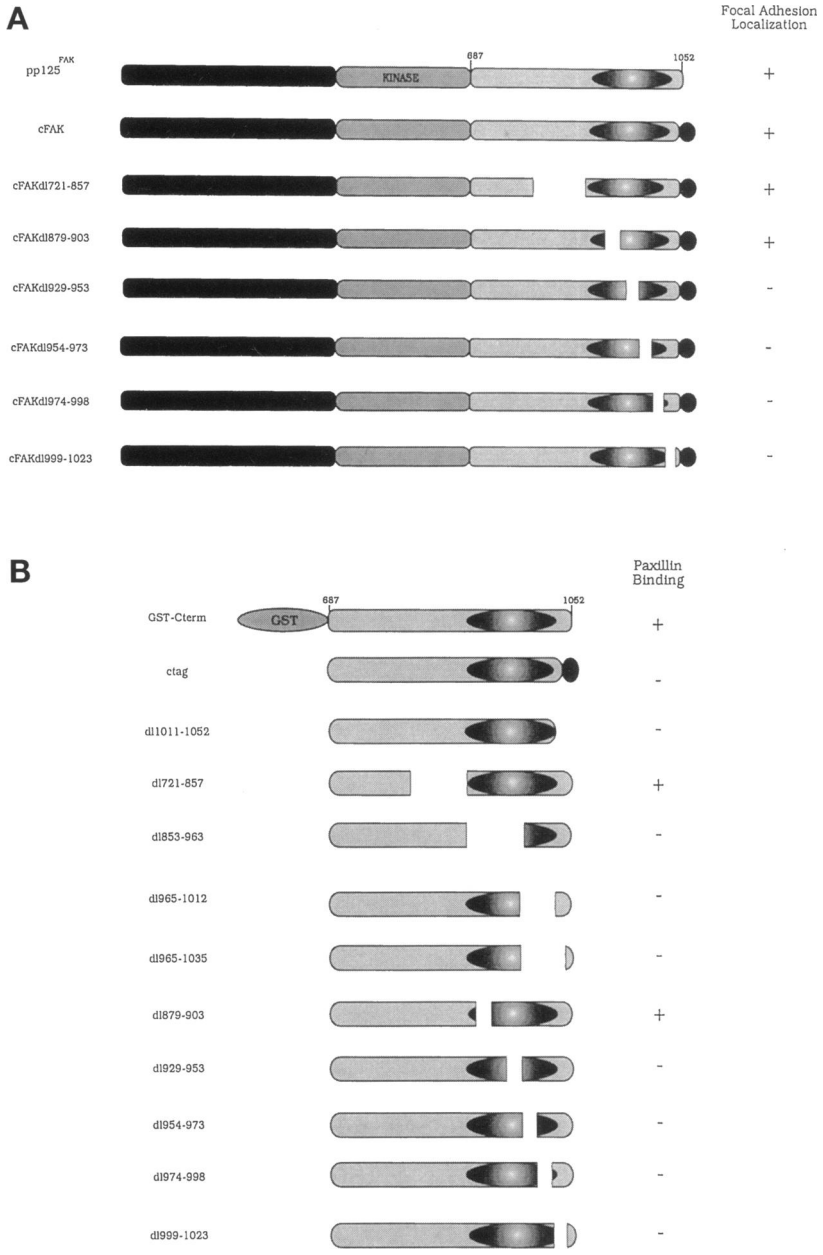

Figure 3. Schematic representation of pp125^{FAK}, GST-FAK fusion proteins, and deletion variants. (A) Structure of wild-type pp125^{FAK} and pp125^{FAK} variants. Shaded oval represents the focal adhesion–targeting sequence (FAT sequence). Black oval represents the presence and position of the epitope tag. (B) Schematic of the carboxy-terminal domain of pp125^{FAK} and relative position of all deletion mutants. The effect of each mutation on the subcellular localization of pp125^{cFAK} in vivo and Cterm binding to paxillin in vitro is designated at the right (+ indicates focal adhesion localization in vivo or formation of a stable complex with paxillin in vitro). Numbering of deletion variants corresponds to the predicted amino acid sequence of full-length chicken pp125^{FAK} (GenBank data base accession number M86656).

lysate using the pp125^{FAK}-specific polyclonal sera BC3, and subsequently assayed by Western blotting for the presence of the epitope tag using mAb KT3. As shown in Figure 6A, all of the mutants contained the epitope tag and were expressed at equivalent levels. These mutants were then characterized for their ability to localize to the correct subcellular compartment by indirect immunofluorescence. CE cells expressing pp125^{cFAK} variants were grown on glass coverslips and stained with the mAb KT3 as described in MATERIALS AND METHODS. As shown in Figure 7, pp125^{cFAK} (A), cFAKdl721–857 (B), and cFAKdl879–903 (C) displayed typical focal adhesion staining (arrowheads), whereas variants cFAKdl929–953 (D), cFAKdl954–973 (E), cFAKdl974–998 (F), and cFAKdl999–1023 (G) exhibited a disperse cytoplasmic staining. In CE cells overexpressing either pp125^{FAK} or pp125^{cFAK}, paxillin subcellular localization appeared to be unaffected (our unpublished results). These data indicate that the FAT sequence is defined by amino acid residues 904 through 1040.

These same FAT sequence deletion mutations were then assayed in the context of the Cterm fusion protein to ascertain their effect on paxillin binding in vitro

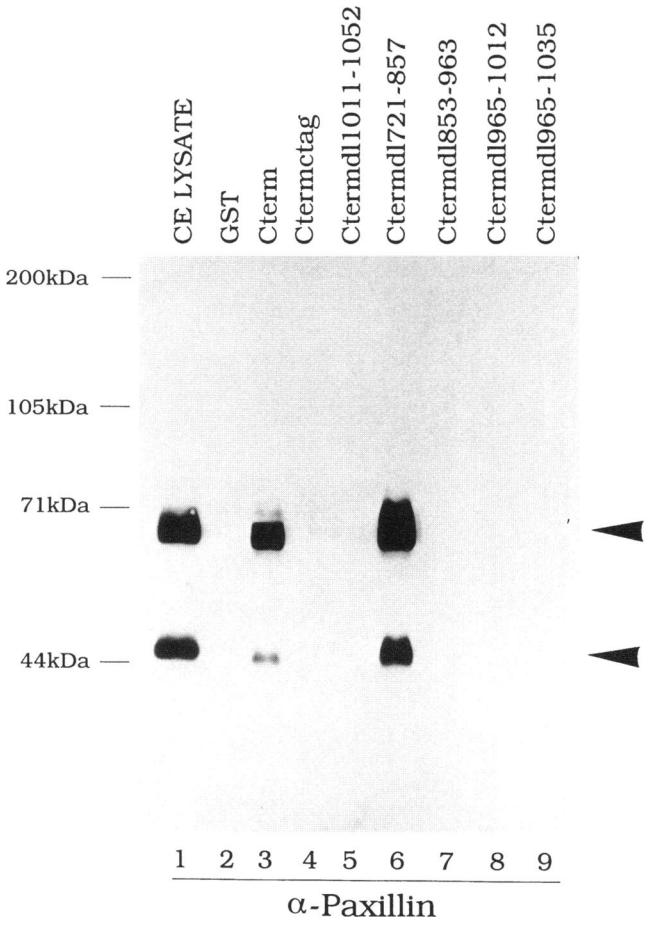

Figure 4. In vitro association of paxillin and pp125^{FAK} carboxy-terminal domain. GST-FAK fusion proteins were incubated with 500 mg CE lysate as described in MATERIALS AND METHODS. Associated proteins were eluted in Laemmli sample buffer and evaluated by Western blotting using a paxillin-specific mAb. To determine the relative efficiency of paxillin association, 50 μg of total cell lysate was evaluated directly (lane 1). Deletion variants are indicated across the top. Arrowheads denote paxillin and the presumed proteolytic fragment of paxillin.

(Figure 8). Only Ctermdl879–903 retained the ability to complex with paxillin in CE lysate (Figure 8, lane 2), whereas Ctermdl929–953, Ctermdl954–973, Ctermdl974–998, and Ctermdl999–1023 were unable to form this complex (Figure 8), indicating that any mutation that perturbs the FAT sequence also compromises paxillin binding.

The FAT Sequence Is Sensitive to Structural Perturbation

We have shown that even small mutations in the FAT sequence abolish both paxillin binding and correct subcellular localization, indicating that paxillin may target pp125^{FAK} to focal adhesions. However, paxillin binding and localization can be separated. One possible explanation for why paxillin binding and focal adhesion targeting activity are difficult to separate is that even small mutations completely disrupt the proper folding of the entire FAT sequence. Support for this hypothesis comes from efforts to map the epitope for the mAb 2A7 (Figure 6). pp125^{FAK}, pp125^{cFAK}, or pp125^{cFAK} deletion mutants were immunoprecipitated using BC3 and the immune complexes were evaluated by Western blotting using either KT3 (Figure 6A) or 2A7 (Figure 6B). As seen in Figure 6, panel A, all of the pp125^{cFAK} variants expressed the epitope tag and were recognized by KT3 whereas wild-type pp125^{FAK} was not recognized. Conversely, only pp125^{FAK}, pp125^{cFAK}, and cFAKdl879–903 were recognized by 2A7 (Figure 6, panel B). These data suggest that the epitope for 2A7 is dependent on the correct folding of the protein. Interestingly, every mutation that ablates 2A7 recognition also effects focal adhesion localization and

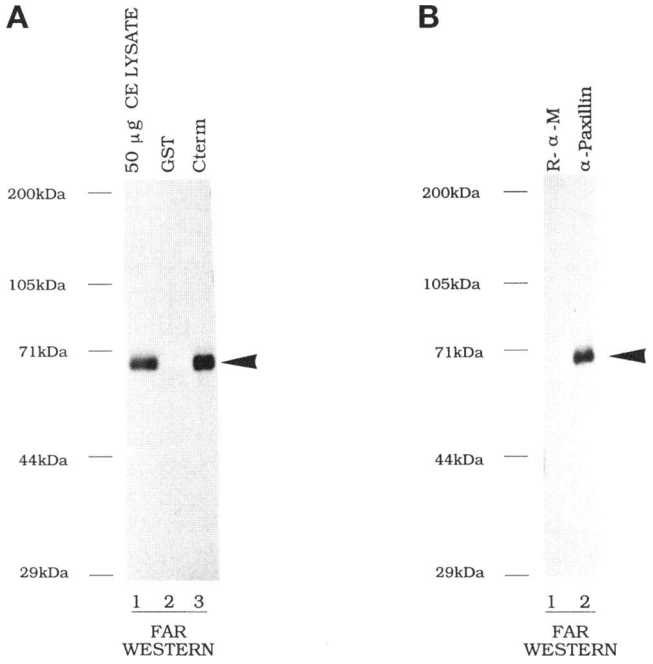

Figure 5. Direct association of paxillin with the pp125^{FAK} carboxy-terminal domain. (A) Farwestern blot of 50 μg CE lysate (lane 1), GST-associated proteins (lane 2), or Cterm-associated proteins (lane 3). For lanes 2 and 3, GST or Cterm immobilized on glutathione Sepharose was incubated with 500 μg cell lysate as described in MATERIALS AND METHODS. Subsequently, associated proteins were eluted in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. (B) Farwestern blot of paxillin immune complexes. Immune complexes were prepared from 1 mg total cell lysate using a paxillin-specific mAb (lane 2) or rabbit anti-mouse (R-α-M) alone as a nonspecific control (lane 1). Immune complexes were subsequently resolved by SDS-PAGE and transferred to nitrocellulose. After transfer, filters corresponding to panels A and B were treated as described in MATERIALS AND METHODS, probed with ^{32}P-labeled Cterm, washed, and exposed to film at −70°C. Arrowheads denote paxillin.

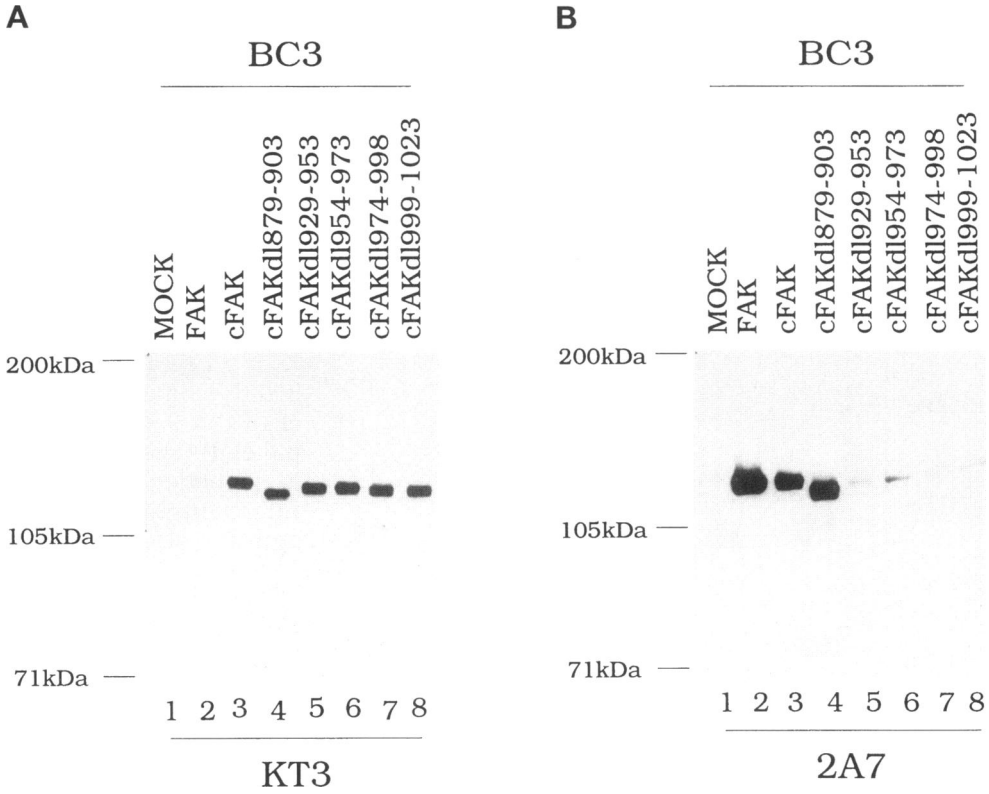

Figure 6. Expression of pp125^{cFAK} deletion mutants and recognition by mAb 2A7. pp125^{cFAK} deletion variants were expressed in CE cells using the retroviral vector RCAS A (BH) and subsequently immunoprecipitated from 500 μg cell lysate using the pp125^{FAK}-specific rabbit polyclonal sera BC3. The precipitates were divided in half and evaluated by Western blotting using either mAb KT3 (A) or mAb 2A7 (B).

paxillin binding. However, the presence of the epitope tag perturbs neither localization nor 2A7 recognition, but does abolish paxillin binding.

DISCUSSION

In this manuscript we present evidence demonstrating that pp125^{FAK} and paxillin form a complex both in vivo and in vitro. We further show that the association is direct and mediated through carboxy-terminal sequences within pp125^{FAK}. The significance of this association is unknown, but we hypothesize that this interaction may serve to align paxillin and pp125^{FAK} and function in the coordination of signals from focal adhesions to the cytoplasm or cytoskeleton.

Paxillin binding is mediated by the carboxy-terminus of pp125^{FAK} because complex formation can be reconstituted in vitro using a bacterially expressed construct encoding only the carboxy-terminal noncatalytic domain of pp125^{FAK}. Deletion analysis of this carboxy-terminal domain has implicated the region spanning residues 904-1054 in paxillin binding. This same region has been defined as the FAT sequence, the sequence that mediates the correct subcellular localization of pp125^{FAK}. However, our analysis also indicates that the paxillin binding site may be distinct from the FAT sequence, because pp125^{cFAK} (containing a carboxy-terminal epitope tag) localizes to focal adhesions but does not stably associate with paxillin. It is possible that the carboxy-terminal domain of pp125^{FAK} mediates binding to several proteins, one of which is paxillin and the other an unidentified protein(s) that anchors pp125^{FAK} to focal adhesions. It is not clear whether the paxillin binding site on pp125^{FAK} truly extends from residue 904 through 1054 or if the engineered deletions perturb the conformation of the carboxy-terminus. Support for the latter hypothesis comes from studies of the mAb 2A7, the epitope for which appears to reside within the FAT sequence. Interestingly, any deletion mutation that perturbs both focal adhesion localization and paxillin binding also abrogates recognition by 2A7. These data suggest that the proper tertiary folding of this domain is crucial for its biological function.

A cDNA encoding the majority of paxillin has recently been cloned and sequenced (Turner and Miller, 1994). The putative amino acid sequence predicts the presence of several interesting motifs. The amino terminal domain contains a proline-rich sequence that conforms to a consensus SH3 binding domain. Dispersed throughout the protein are several tyrosine residues that may serve as binding sites for SH2 domains when phosphorylated. Of particular importance are tyrosines 26 and 113, which lie in consensus binding motifs for Crk SH2 domains (Songyang *et al.*,

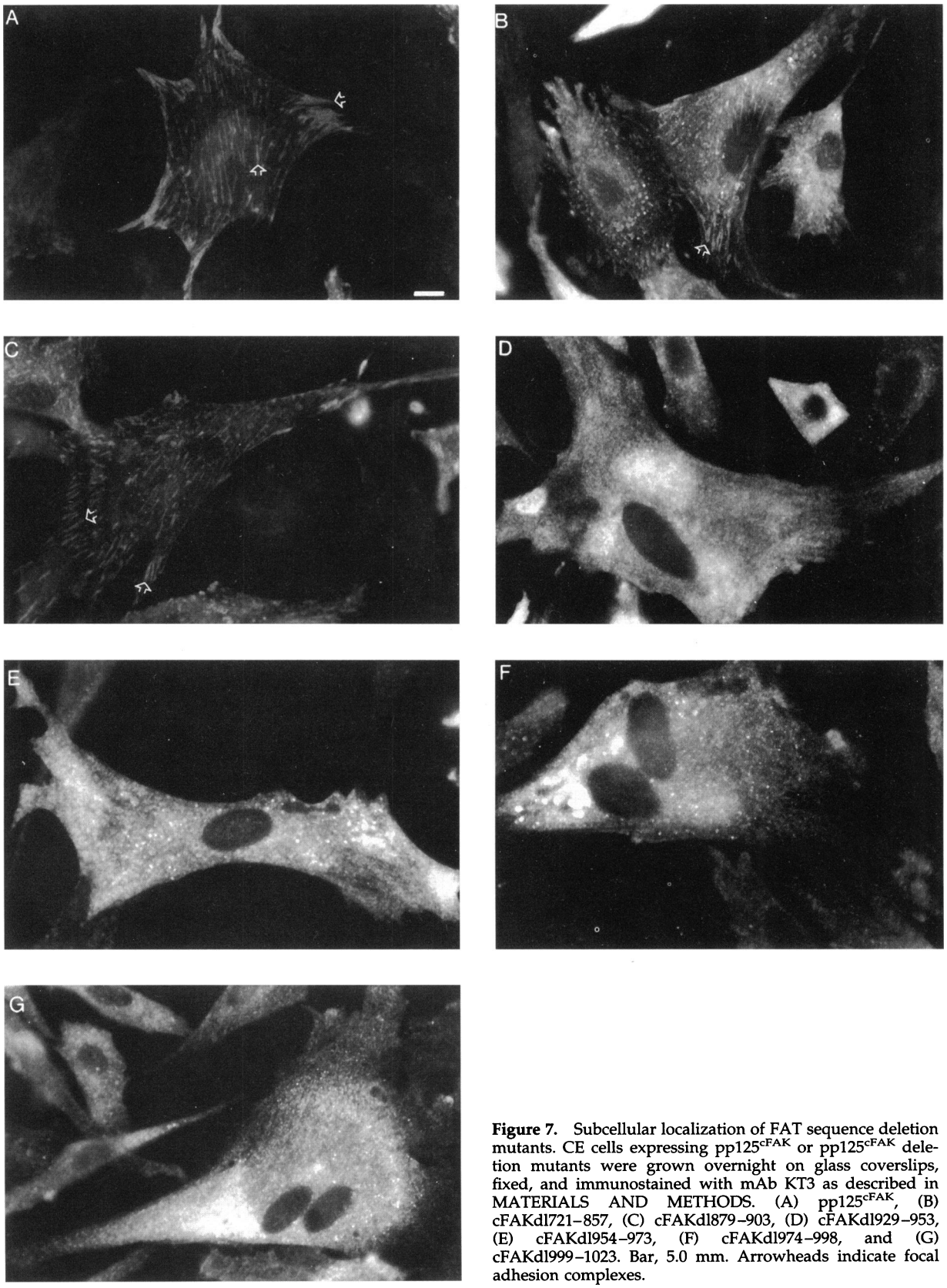

Figure 7. Subcellular localization of FAT sequence deletion mutants. CE cells expressing pp125^{cFAK} or pp125^{cFAK} deletion mutants were grown overnight on glass coverslips, fixed, and immunostained with mAb KT3 as described in MATERIALS AND METHODS. (A) pp125^{cFAK}, (B) cFAKdl721–857, (C) cFAKdl879–903, (D) cFAKdl929–953, (E) cFAKdl954–973, (F) cFAKdl974–998, and (G) cFAKdl999–1023. Bar, 5.0 mm. Arrowheads indicate focal adhesion complexes.

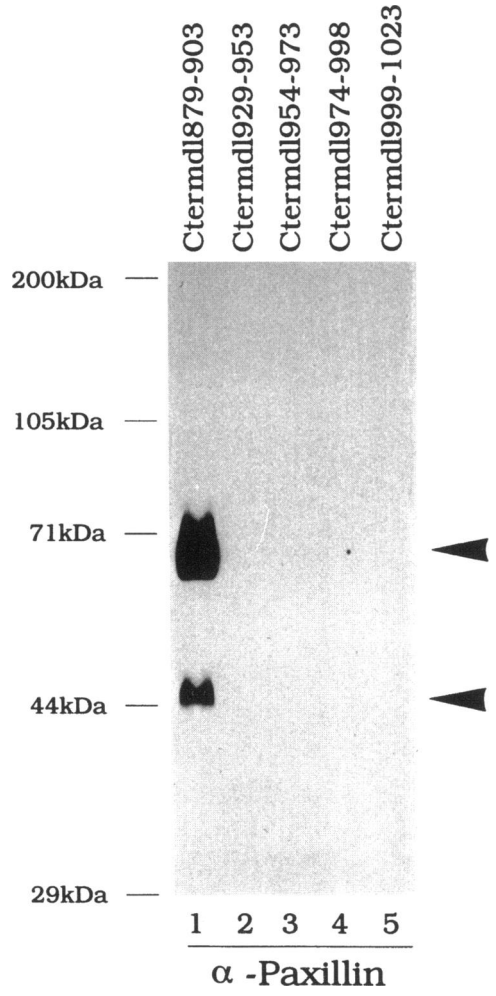

Figure 8. In vitro association of paxillin with FAT sequence deletion mutants. Variants of Cterm containing deletions within the FAT sequence were incubated with 500 μg of precleared CE lysate as described in MATERIALS AND METHODS. The protein complexes were subsequently evaluated by Western blotting using a paxillin-specific mAb. Deletion variants are indicated across the top. Arrowheads denote paxillin and the presumed proteolytic fragment of paxillin.

1993). The carboxy terminal one-third of the protein is comprised of a series of four LIM domains, protein domains that have recently been implicated in mediating protein-protein interactions between other focal adhesion proteins (Schmeichel and Beckerle, 1994). Finally, the amino terminal portion of the protein appears to mediate the association of paxillin with both pp125^{FAK} and vinculin in vitro (Turner and Miller, 1994).

The correct subcellular localization of paxillin may be mediated by one or more cytoskeletal proteins. Paxillin recruitment to focal adhesions may be dependent upon binding to an individual protein, such as vinculin, pp125^{FAK}, or an as yet unidentified cytoskeletal protein, or by multiple, coordinated interactions, such as simultaneous binding to vinculin and pp125^{FAK}. Also, post-translational modifications of either paxillin itself or its binding proteins may play a critical role in paxillin localization, with protein phosphorylation being a candidate regulatory mechanism. It is currently unknown what, if any, effect the binding of pp125^{FAK} to paxillin has on the ability of pp125^{FAK} to phosphorylate paxillin.

Recent evidence indicates that paxillin may function as an adapter protein serving to mediate the formation of multicomponent protein complexes. Paxillin has been shown to bind to the SH2 domains of the small adapter protein p47$^{gag-crk}$ (Birge *et al.*, 1993) and the protein tyrosine kinase Csk (Sabe *et al.*, 1994), to the SH3 domain of pp60^{src} (Weng *et al.*, 1993), and now to pp125^{FAK}. By mediating the formation of extensive protein complexes, paxillin may help coordinate the transmission of cytoplasmic signals from focal adhesions. The pp125^{FAK}/c-Src/Csk/paxillin complex may function as a multimeric enzyme complex with both positive and negative regulatory components. It is well documented that paxillin becomes tyrosine phosphorylated after a variety of cellular stimuli (Glenney and Zokas, 1989; Burridge *et al.*, 1992; and Turner *et al.*, 1993; Zackary *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994). In vitro experiments indicate that different kinases (e.g., Csk and Src) appear to phosphorylate paxillin on distinct sites (Schaller and Parsons, 1995), potentially creating several distinct binding sites for specific SH2-containing signaling molecules. Thus, differential phosphorylation of paxillin by kinases such as pp125^{FAK}, pp60^{src}, or Csk, could be converted into a variety of cytoplasmic signals, providing yet another level of regulation upon integrin signaling.

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