A Fragment of Paxillin Binds the $\alpha_4$ Integrin Cytoplasmic Domain (Tail) and Selectively Inhibits $\alpha_4$-Mediated Cell Migration*

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The $\alpha_4$ integrins play important roles in embryogenesis, hematopoiesis, cardiac development, and the immune responses. The $\alpha_4$ integrin subunit is indispensable for these biological processes, possibly because the $\alpha_4$ subunit regulates cellular functions differently from other integrin $\alpha$ subunits. We have previously reported that the $\alpha_4$ cytoplasmic domain directly and tightly binds paxillin, an intracellular signaling adaptor molecule, and this interaction accounts for some of the unusual functional responses to $\alpha_4$ integrin-mediated cell adhesion. We also have identified a conserved 9-amino acid region (Glu983-Tyr991) in the $\alpha_4$ cytoplasmic domain that is sufficient for paxillin binding, and an alanine substitution at either Glu983 or Tyr991 within this region disrupted the $\alpha_4$-paxillin interaction and reversed the effects of the $\alpha_4$ cytoplasmic domain on cell spreading and migration. In the current study, we have mapped the $\alpha_4$-binding site within paxillin using mutational analysis, and examined its effects on the $\alpha_4$-tail-mediated functional responses. Here we report that sequences between residues Ala176 and Asp275 of paxillin are sufficient for binding to the $\alpha_4$ tail. We found that the $\alpha_4$ tail, paxillin, and FAT, the focal adhesion targeting domain of pp125FAK, could form a ternary complex and that the $\alpha_4$-binding paxillin fragment, P(Ala176–Asp275), specifically blocked paxillin binding to the $\alpha_4$ tail more efficiently than it blocked binding to FAT. Furthermore, when expressed in cells, this $\alpha_4$-binding paxillin fragment specifically inhibited the $\alpha_4$ tail-stimulated cell migration. Thus, paxillin binding to the $\alpha_4$ tail leads to enhanced cell migration and inhibition of the $\alpha_4$-paxillin interaction selectively blocks the $\alpha_4$-dependent cellular responses.

Integrins are a large family of transmembrane adhesion receptors that each is composed of a $\alpha$ and a $\beta$ subunit (1–3). Integrins mediate cell adhesion and cell migration, and regulate gene expression and cell survival (1, 3). The $\alpha_4$ integrins are primarily expressed on various leukocytes and play important roles in embryogenesis, hematopoiesis, cardiac development, and the immune responses (4–7). The $\alpha_4$ integrin subunit is indispensable for these biological processes, possibly because the $\alpha_4$ subunit regulates cellular functions differently from other integrin $\alpha$ subunits. Indeed, the $\alpha_4$ integrin promotes increased cell migration and less cell spreading and focal adhesion formation relative to most other $\beta_1$ integrins. These unusual functional properties are mediated by the $\alpha_4$ cytoplasmic domain (8, 9) because this region of $\alpha_4$ markedly stimulates cell migration, and opposes cell spreading and focal adhesion formation when joined to other integrin $\alpha$ subunits (9–11).

We previously reported that the $\alpha_4$ cytoplasmic domain directly and tightly binds paxillin, an intracellular signaling adaptor molecule (10, 11). The $\alpha_4$-paxillin interaction accounts for some of the unusual functional responses to $\alpha_4\beta_1$ integrin-mediated cell adhesion, including stimulating cell migration and opposing cell spreading and focal adhesion formation (10, 11). We have identified a conserved 9-amino acid region (Glu983-Tyr991) in the $\alpha_4$ cytoplasmic domain that is sufficient for paxillin binding (11), and an alanine substitution at either Glu983 or Tyr991 within this region disrupted the $\alpha_4$-paxillin interaction and reversed the effects of the $\alpha_4$ cytoplasmic domain on cell spreading and migration (10, 11).

Paxillin is a 68-kDa cytoplasmic protein that is involved in cellular responses to integrin-dependent adhesion (12, 13). Paxillin has the structural properties of a signaling adaptor molecule. It contains four C-terminal LIM protein-protein interaction motifs that serve to target it to focal adhesions (12, 13), and five N-terminal LD motifs that mediate protein-protein interactions (12–14). Paxillin directly interacts with several cytoskeletal, intracellular signaling, and adaptor molecules such as Src, PTP-PEST, Crk, p95 PKL, actopaxin, and ILK (15–22). Paxillin also interacts with pp125FAK, a molecule strongly implicated in the regulation of cell migration (23, 24), by binding to a C-terminal domain of pp125FAK termed the focal adhesion targeting (FAT) domain (Fig. 1A). Furthermore, the $\alpha_4$ cytoplasmic domain markedly enhances activation of pp125FAK. The enhanced pp125FAK phosphorylation depends on the integrity of the paxillin-binding site in the $\alpha_4$ tail (10). These results suggest that the direct association of paxillin with the $\alpha_4$ cytoplasmic domain might facilitate the rapid re-

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Enzyme-linked Immunosorbent Assays—Wells of Ni-NTA HisSorb strips (Qiagen) were coated with 100 μl of His-tagged integrin model proteins or His-tagged FAT, a recombinant protein derived from the focal adhesion targeting sequence of focal adhesion kinase (FAK), dissolved in phosphate-buffered saline (PBS) plus 0.01% BSA at 4 °C overnight. The next day, wells were washed with PBS three times, blocked with 150 μl of 1% (w/v) heat-denatured BSA at room temperature for 1 h. The wells were then washed with PBS three times. 100 μl of recombinant paxillin or its mutants at different concentrations dissolved in PBS plus 0.2% (w/v) BSA was added to each well and incubated at room temperature for 1 h. Unbound proteins were washed out with PBS three times. Bound proteins were stained with mouse anti-HA tag (1:1,000 dilution in PBS plus 1% BSA) or mouse anti-GST antibodies (1:1,000 dilution in PBS plus 1% BSA) for 1 h at room temperature, followed by 1 h incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000 dilution in PBS plus 1% BSA) (BioSource). After three washes, bound proteins were assayed by measuring peroxidase activity with o-phenylenediamine as a substrate and quantified by reading its optical density at 490 nm. For competition assays using paxillin fragment, P(Ala176→Asp275), or recombinant full-length paxillin (GST-free), the same modified ELISA assays were performed except that different concentrations of this fragment was included in the paxillin solution added to the integrin model protein or FAT-coated wells. Data were expressed as percentage of inhibition: \( (1 - \beta/B) \times 100\% \), where \( B \) is the absorbance in the presence of the competitor and \( B_0 \) is the absorbance in its absence.

cDNA Construction, Transfection, and Expression of Paxillin Mutants, Immunoprecipitation, and Western Blotting—For construction of mammalian expression vectors encoding paxillin fragments, PCR was used to generate an Xhol-HindIII fragment including Myc tag, EQKLI-SEEDL, sequence at the 3’ end of each paxillin fragment sequence. Each PCR product was ligated into the pCR vector using a TA cloning kit (Invitrogen). After confirmation by DNA sequencing, each fragment was ligated into Xhol-HindIII sites of pcDNA3.1+ vector (Invitrogen). \( \alpha_1\)β2,β3, or \( \alpha_4\)β4-expressing CHO cells were co-transfected with vector encoding each paxillin fragment plus vector encoding GFP (cDNA ratio of paxillin fragment to GFP, 50:1), or a control vector plus GFP at the same ratio, using LipofectAMINE transfection (LipofectAMINE PLUS, Invitrogen) following the manufacturer’s instructions. Forty-eight hours after transfection, the cells were trypsinized and resuspended. Aliquots of cells were used for cell adhesion or migration as described below, and other cells were lysed using RIPA buffer. Expression of each paxillin fragment was detected by Western blot analysis on the cell lysate using a monoclonal antibody specific for Myc tag (9E10) or polyclonal antibodies specific for paxillin. Immunoprecipitation was performed as described previously (10, 11). Briefly, for co-precipitation of the α1 integrin with the α1-binding paxillin fragment, cell lysate from α1α1β2β3-expressing CHO cells transiently transfected with a P(Ala176→Lys275) construct was precipitated using a monoclonal antibody specific for Myc tag (9E10). The precipitated proteins were detected by Western blot analysis using polyclonal antibodies specific against the α1a1 cytoplasmic domain. The cells were then stripped and blotted with polyclonal antibodies specific for paxillin. For co-precipitation of the α4 integrin, paxillin, and p125FAK, cell lysate from α4β4-expressing CHO cells was precipitated with polyclonal antibodies specific for p125FAK (10). Co-precipitation of the intact α4 integrin, paxillin, and p125FAK was detected using polyclonal antibodies against the α4 cytoplasmic domain (28) or polyclonal (C-20, Santa Cruz), and a monoclonal antibody specific for paxillin (clone 349, Transduction Biosciences).

Cell Adhesion and Migration Assays—Assays of cell adhesion and migration on fibrinogen or fibronectin (FN) were performed as described previously (10, 11). Briefly, for cell adhesion assay, 24-well plates were coated with 10 μg/ml fibrinogen or FN in a coating buffer: NaCl, 150 mM; NaHPO4, 50 mM; and Na2HPO4, pH 8.0, at 4 °C overnight and blocked with 1% heat-denatured BSA at 37 °C for more than 1 h. Equal numbers of α1α1β2β3-expressing CHO cells transfected with different cDNA constructs as described were plated on the fibrinogen- or FN-coated wells and incubated in a 37 °C incubator for 30 min. At the end of the experiment, unattached cells were washed away with PBS. Attached cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and counted under a microscope with high magnification.

For cell migration using Transwell chambers (8 μm, Costar), both sides of chambers were coated with 10 μg/ml fibrinogen or FN overnight at 4 °C. The coated chambers were blocked with 1% heat-denatured BSA, 100 μl of transfected cells (1.0 × 106 cells) resuspended in Dulbecco’s modified Eagle’s medium plus 0.5% FBS were added to the

**Interaction Selectively Blocks**

**this idea by examining the capacity of the**

\( \alpha_4 \)-integrins. In the current study, we have tested this idea by examining the capacity of the **α4** tail to form ternary complex with paxillin and p125FAK. Furthermore, we have mapped the **α4**-binding site to a 100-amino acid fragment within the N-terminal domain of paxillin. This **α4**-binding fragment blocked the binding of paxillin to the **α4** tail to a much greater extent than to FAT. Furthermore, when expressed in cells, this **α4**-binding paxillin fragment inhibited **α4** tail-stimulated cell migration, but not migration mediated by integrin **α2β1**. Thus, the binding of paxillin to the **α4** tail leads to enhanced cell migration and specific inhibition of **α4**-paxillin interaction selectively blocks **α4**-dependent cellular responses.

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**Chinese hamster ovary (CHO) cells expressing α1β2β3 chimeric integrin or α1β3 integrin have been described previously (10, 11, 29). These cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% non-essential amino acids (Sigma), 50 units of penicillin/ml, and 50 μg of streptomycin sulfate/ml in a 37 °C tissue culture incubator. The following antibodies were obtained commercially: monoclonal antibodies against HA-tag (12CA5, American Type Culture Collection), against GST (B-14, Santa Cruz), and against Myc-tag (9E10, Santa Cruz); polyclonal antibodies against α1β2 (C-20, Santa Cruz). Polyclonal antibodies against the α4 cytoplasmic domain have been described previously (29).

**Integrin Cytoplasmic Domain Model Proteins, Recombinant Paxillin Mutant Proteins, and Binding of Paxillin Mutants to Model Proteins—**The design and production of recombinant wild-type integrin cytoplasmic domain proteins have been described (11, 25). Each recombinant protein model was expressed in BL21 (DE3) (PlysS cells (Novagen), isolated by Ni2+-charged resins, and further purified to >90% homogeneity using a reverse-phase C18 high performance liquid chromatography column (Vydac). Masses of all proteins were assessed by electrospray ionization mass spectrometry on an API-III quadrupole spectrometer (Sciex, Toronto, Canada) and varied by less than 0.1% from the predicted mass.

**Expression and isolation of recombinant glutathione S-transferase (GST) fusion protein of wild-type paxillin, mutants of P(Ile43→Gly60), P(Ala57→Asp60), P(Gln191→Glu216), PYS31YA118Y181A, N-terminal domain, P(Met4→Gly315), and C-terminal LIM domain, P(Gly315→Cys325), have been described previously (26, 27). Recombinant full-length paxillin (GST-free) was produced by thrombin digestion of recombinant GST-paxillin fusion protein and purification through glutathione-Sepharose (Amersham Biosciences).**

**Four truncation mutants of N-terminal domain of paxillin were created by site-directed mutagenesis using the QuickChange kit (Stratagene). Primers for QuickChange reactions were designed so that at each truncation site, the amino acid codon was replaced with a stop codon. Polymerase chain reaction (PCR) was performed using wild-type GST-paxillin cDNA construct as a template following the manufacturer’s instruction. Each site-directed mutation was then confirmed by cDNA sequencing, and expression and isolation of the mutant protein were performed as described (11, 26). For construction of other paxillin mutants, PCR was used to generate a BamHI-XhoI fragment for each mutant. Each PCR product was ligated into the pCR vector using a TA cloning kit (Invitrogen). After cDNA sequencing, each fragment was ligated into BamHI-XhoI sites of pGEX-4T-3 vector (Amersham Biosciences), and expression and isolation of each GST fusion protein were performed as described (11, 26).

**Binding of recombinant paxillin or its mutants to integrin tail model proteins was performed as described (11, 25). Briefly, aliquots of recombinant GST fusion protein of paxillin or its mutants were mixed with 300 μl of buffer A: 10 mM Pipes, 50 mM NaCl, 150 mM sucrose, 1 mM Na2VO4, 50 mM NaF, 40 mM sodium pyrophosphate, pH 6.8, plus 0.5% sodium deoxycholate, 1 mM EDTA, 20 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.1% Triton X-100, 0.5 mM MgCl2, and 1 mg/ml bovine serum albumin (BSA), added to 20 μl of model protein-loaded resins, and incubated at room temperature with rotation for 2 h. Resins were then washed three times with the same buffer. Bound proteins were extracted with reducing SDS sample buffer, separated on SDS-polyacrylamide gels (PAGE), and detected with antibodies specific for HA-tag or GST followed by ECL (Amersham Biosciences).**
upper chamber and 500 μl of same medium added to the lower chamber. The cells were then allowed to migrate at 37 °C for 4 h. At the end of the experiment, the cells migrated to the lower side were collected and counted either under a microscope with high magnification or counted using fluorescence-activated cell sorting analysis.

For cell migration assay using real time video phase-contrast microscopy, cells (2.0 × 10^4) were plated on coverslips coated with 10 μg/ml fibronogen or FN. Dishes for cell migration were prepared as described previously (30). Dishes were placed in an open chamber with atmospheric and temperature control and cell movement viewed with a Nikon DiaPhot Microscope equipped with a SenSys cooled CCD video camera linked to a Silicon Graphics work station running the Inovision ISEE software program.

Fibroblast and temperature control and cell movement viewed with a Nikon DiaPhot Microscope equipped with a SenSys cooled CCD video camera linked to a Silicon Graphics work station running the Inovision ISEE software program.

**RESULTS**

**Mapping of the α4 Integrin Cytoplasmic Domain Binding Site in Paxillin**—Paxillin binding to the α4 cytoplasmic domain accounts for some of unusual biological properties of the α4 integrins (10, 11, 29). We employed integrin tail model protein affinity chromatography to identify the regions of paxillin responsible for binding to the α4 cytoplasmic domain. The N-terminal half of paxillin, P(Met1-Gly326), bound to the α4 cytoplasmic tail to the same extent as the full-length protein (Fig. 1, B and C). In contrast, the C-terminal half, comprised of four LIM domains, P(Gly326-Cys557), failed to bind (Fig. 1, B and C). Thus, the N-terminal region of paxillin is necessary and sufficient for paxillin binding.

The N-terminal half of paxillin contains several regions known to mediate protein-protein interactions. This includes a Pro-rich domain responsible for its interaction with the SH3 domains of Src and Crk family members (31), however, removal of this domain, P(A/Ile43-Gly101) (Fig. 1B), was without effect on binding to the α2 tail. The N terminus of paxillin also contains 5 LD repeats known to be involved in its interactions with binding partners (14). An internal deletion that disrupts LD repeats LD2 and LD3, P(A/Glu101-Glu226), partially blocked binding to the α2 tail (Fig. 1, B and C). In contrast, a further N-terminal half, P(A/Glu101-Glu226), did not abolish α2 binding activity (Fig. 1, B and C). In addition, paxillin contains multiple tyrosines that can become phosphorylated to mediate binding to Crk adaptors or Csk kinase (12, 13). However, alanine substitutions at these Tyr residues, P(Y31A,T118A, T181A), did not affect the α2 binding (Fig. 1, B and C). These data indicate that the α2 binding function of paxillin can be separated from many of its other binding activities and that residues contained in the Glu101–Glu226 interval contribute to this activity.

To further narrow the localization of the paxillin-binding site, we analyzed sequential C-terminal truncation mutants of the N-terminal half of paxillin. P275X bound to the α4 tail, suggesting that the last 50 amino acid residues of N-terminal region of paxillin are dispensable for the α4 binding (Fig. 2). However, removal of 50 more residues (P225X) markedly reduced the binding to ~25% of that of N terminus. An additional 50-residue truncation (P175X) blocked binding completely (Fig. 2). Thus, these data show that sequences between residues Ala176–Asp275 are required for paxillin to bind the α4 tail.

The foregoing studies identified a 100-residue sequence required for paxillin binding to the α4 tail. To determine whether sequences from this region were sufficient for α4 binding, we assessed the capacity of a fragment containing these residues, P(Ala176–Asp275), to bind to the α4 tail. This fragment bound the α4 tail, but to a lesser extent than the complete N terminus of paxillin, P(1–315) (Fig. 3). In contrast, smaller fragments P(Glu226–Cys325), P(Ala176–Glu226), P(Phe276–Asp275), and P(Phe276–Cys325) were nearly devoid of activity (Fig. 3 and data not shown). In addition, individual LD domain, i.e. LD1 to LD5, revealed very weak binding to the α4 tail that was similar to that of P(Phe276–Cys325) (data not shown). Thus, each of the LD repeats may contribute to the binding of paxillin to the α4 tail, accounting for the reduced affinity of P(Ala176–Asp275) relative to the intact protein. However, the residues contained between Ala176 and Asp275 are sufficient for detectable paxillin binding to the α4 tail.

**FIG. 1.** The N-terminal region of paxillin contains a binding site for the α4 integrin cytoplasmic domain. A, schematic presentation of paxillin structure. B, the schematic presentation of each paxillin mutant is illustrated (top panel). Recombinant wild-type or mutant paxillin GST fusion protein was added to Ni2⁺-charged resins loaded with α4 or αm, (data not shown) model proteins. Bound fractions were collected and separated on 4–20% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with antibodies specific for GST. The quantity of each bound protein was estimated by scanning densitometry using the NIH Image program and expressed as a percentage of starting material for each construct that bound to the α4 tail (B, right column, and C).
**a4 Integrin-Paxillin Interaction**

**Fig. 2.** Paxillin(Ala\(^{176}\)-Asp\(^{275}\)) is required for binding to the a4 tail. Each recombinant paxillin N-terminal domain truncation GST fusion protein (A) was added to Ni\(^{2+}\)-charged resins loaded with a4 or \(\alpha_{IIb}\) model proteins. Bound fractions were collected and separated on 4–20% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with antibody specific for GST (B, top panel). Loading of each paxillin mutant protein was assessed by Coomassie Blue staining (B, bottom panel). The quantity of binding of each mutant was estimated by scanning densitometry using the NIH Image program and expressed as a percentage of starting material for each construct that bound to the a4 tail (C). None of the proteins bound to the \(\alpha_{IIb}\) model protein (data not shown).

**Fig. 3.** Paxillin(Ala\(^{176}\)-Asp\(^{275}\)) binds to the a4 tail. Each recombinant paxillin N-terminal domain GST fusion protein (A) or GST only (data not shown) was added to Ni\(^{2+}\)-charged resins loaded with a4 or \(\alpha_{IIb}\) model proteins. Bound fractions were collected and separated on 4–20% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with antibody specific for GST (B, top panel). Loading of each paxillin mutant protein was assessed by Coomassie Blue staining (B, bottom panel). The quantity of binding of each mutant was estimated by scanning densitometry using the NIH Image program and expressed as a percentage of starting material for each construct that bound to the a4 tail (A, right column).

\(\alpha_{IIb}\), Paxillin, and the FAT Domain of pp125FAK Can Form a Ternary Complex. We previously hypothesized that the \(\alpha_{IIb}\)-paxillin and pp125FAK form a ternary complex leading to the increased membrane targeting and clustering of pp125FAK and rapid pp125FAK phosphorylation (10). To directly test this idea, we used affinity chromatography to examine the interactions among the a4, paxillin, and FAT, a fragment of pp125FAK which contains its paxillin-binding site (32). Paxillin directly bound to the a4 tail, whereas FAT did not show detectable direct binding (Fig. 4A). In contrast, in the presence of paxillin, FAT binding was detected. Neither paxillin nor FAT bound to the \(\alpha_{IIb}\) tail (Fig. 4A). Thus, FAT does not directly bind to the a4 tail but it does interact with the a4 tail through paxillin. Furthermore, the presence of FAT, even at a 100-fold molar excess, did not inhibit paxillin binding or lead to increased FAT binding to the a4 tail (Fig. 4B and data not shown). In addition, using the ELISA assay that we developed (see “Materials and Methods”), we were also able to demonstrate the formation of a ternary complex of the a4 tail, paxillin, and FAT (data not shown). Therefore, the a4 integrin tail, paxillin, and FAT can form a ternary complex. To test whether the intact a4 integrin, paxillin, and pp125FAK also form a ternary complex in vivo, we performed co-precipitation experiments. As shown in Fig. 4C, both the a4 integrin and paxillin co-precipitated with the pp125FAK. Thus, the intact a4 integrin, paxillin, and pp125FAK can also form a ternary complex in cells.

P(Ala\(^{176}\)-Asp\(^{275}\)), an \(\alpha_{IIb}\)-Binding Fragment of Paxillin, Blocks Paxillin Binding to the a4 Tail More Efficiently Than to the Focal Adhesion Targeting Sequence of pp125FAK. To further characterize the interactions of paxillin with a4 and FAT, we developed a quantitative ELISA assay. In the assay, the a4 tail model protein or FAT were immobilized on Ni\(^{2+}\)-charged resins and the immobilized paxillin(Ala\(^{176}\)-Asp\(^{275}\)) completely blocked GST-paxillin binding (Fig. 5A). Binding was specific because no interaction was detected with immobilized \(\alpha_{IIb}\) tail and a recombinant full-length paxillin (GST-free) completely blocked GST-paxillin binding to the a4 tail (Fig. 5, A and D). Similarly, in the ELISA assays, paxillin binding to FAT was specific and saturable (Fig. 5C). The a4 binding 100-residue fragment, P(Ala\(^{176}\)-Asp\(^{275}\)), bound with a reduced affinity (EC\(_{50}\) ~27 nM, Fig. 5B). Thus, both paxillin and the P(Ala\(^{176}\)-Asp\(^{275}\)) bind tightly to the a4 tail. Since P(Ala\(^{176}\)-Asp\(^{275}\)) contains both LD3 and LD4 motifs, in which LD4 motif has been shown to mediate pp125FAK-paxillin interaction (20), it is possible that this fragment might also interfere with the pp125FAK-paxillin interaction. To deter-
FIG. 4. Paxillin forms a ternary complex with the α4 integrin tail and FAT. Recombinant FAT, HA-tagged-paxillin-GST, or a mixture of both proteins were added to Ni²⁺-charged resins loaded with α4 or αIIb, model proteins. Bound fractions were collected and separated on 4–20% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with antibody specific for FAK (A, top panel). The membrane was then stripped and re-stained with antibody specific for HA-tagged paxillin-GST, 12CA5 (A, middle panel). Loading of each tail was assessed by Coomassie Blue staining (A, bottom panel). B, recombinant HA-tagged paxillin-GST was added to Ni²⁺-charged resins in the absence or presence of FAT at the indicated concentration. Bound fractions were collected and separated on 4–20% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with antibody specific for HA-tag (A and C) or GST-paxillin(Ala176–Asp275) as described under “Materials and Methods.” C, cell lysate from the α4,β1-expressing CHO cells was immunoprecipitated using antibodies specific for pp125FAK (α-FAK) or a control rabbit IgG (IgG) as described under “Materials and Methods.” The precipitated proteins were separated on 4–20% SDS-PAGE and detected with antibodies specific for the α4 cytoplasmic domain, paxillin, and pp125FAK, respectively.

FIG. 5. Quantification of the binding of paxillin to the α4 integrin tail or to FAT by ELISA. Recombinant HA-tagged paxillin-GST (A and C) or GST-paxillin(Ala176–Asp275) was added to each well of Ni-NTA HisSorb strips coated with α4 or αIIb model proteins (A and B) or with FAT (C). Bound paxillin was detected with an antibody specific for HA-tag (A and C), or GST (B) as described under “Materials and Methods.” D, recombinant HA-tagged paxillin-GST was added to wells of Ni-NTA HisSorb strips coated with α4 model protein in the absence or presence of different amounts of full-length paxillin (GST-free) as indicated. Bound paxillin was detected with an antibody specific for GST as described under “Materials and Methods.” The same experiment was performed using GST as a competitor. Even at 20-fold molar excess, no significant inhibition on GST-paxillin binding to the α4 tail by GST was observed (data not shown).
mine whether P(Ala176–Asp275) competes for paxillin binding to α4 or to FAT, we performed the paxillin-binding ELISA assays in the presence of the P(Ala176–Asp275) fragment. The P(Ala176–Asp275) fragment effectively competed for paxillin binding to α4, producing 50% inhibition in the presence of 10-fold molar excess of the fragment (Fig. 6A). A 20-fold molar excess of P(Ala176–Asp275) blocked binding by more than 95% (Fig. 6A). In sharp contrast, P(Ala176–Asp275) had a much weaker effect on paxillin binding to FAT. At 10-fold excess, P(Ala176–Asp275) did not have a significant inhibitory effect on paxillin binding to FAT (Fig. 6A). Even at 100-fold molar excess, this fragment only inhibited paxillin binding by ~30% (Fig. 6A). In contrast, at 6-fold molar excess, the full-length paxillin (GST-free) completely inhibited GST-paxillin binding to FAT (Fig. 6B). Thus, P(Ala176–Asp275) specifically blocked paxillin binding to the α4 tail more efficiently than it blocked binding to FAT.

The α4-Binding Fragment of Paxillin Specifically Inhibits α4 Tail-dependent Cell Migration—The previous finding that a mutation of the α4 tail that blocks paxillin binding reduces cell migration (10) suggests that inhibitors of paxillin binding to α4 could perturb α4-dependent cell migration. To test this idea, we transfected cells with plasmids encoding P(Ala176–Lys277), the fragment that blocked α4-paxillin interactions with minimal effects on interactions with pp125FAK. We used CHO cells expressing a chimeric integrin, αIIbβ3β2β4, that contains the α4 cytoplasmic domains in place of that of αIIb (10, 11). The presence of the α4 cytoplasmic domain promotes cell migration and inhibits cell spreading when the cells adhere to an αIIbβ3 ligand, fibrinogen (10, 11). Expression of P(Ala176–Lys277) markedly inhibited cell migration on fibrinogen (Fig. 7A, left panel). Furthermore, expression of P(Ala176–Lys277) fragment also reversed the inhibition of cell spreading by the α4-paxillin interaction. In contrast, expression of a fragment of paxillin that failed to bind to the α4 tail, P(Met1–Lys125), did not inhibit cell migration on fibrinogen (Fig. 7A, left panel). Importantly, both fragments were well expressed (Fig. 7B). Interestingly, the inhibitory fragment was expressed at a level only 3–4-fold greater than that of endogenous paxillin, yet it dramatically reduced cell migration. The inhibition of cell migration by the α4-binding paxillin fragment was associated with its binding to the integrin, since the αIIbβ3β2β4 chimeric integrin co-precipitated with the α4-binding paxillin fragment, P(Ala176–Asp275) (Fig. 7C). Thus, expression of a paxillin fragment that binds to the α4 tail and disrupts its interaction with paxillin inhibited cell migration.

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migration mediated by the α4 cytoplasmic domain.

As noted above, P(Ala176–Lys277) was much less effective at blocking the binding of paxillin to FAT. The paxillin-FAR interaction may be involved in cell migration mediated by many classes of integrins, suggesting that P(Ala176–Lys277) would not efficiently inhibit migration mediated by integrins other than αβ1 and αβ12. To test this idea, we examined the effect of this fragment on cell migration on fibronectin (FN), which is mediated by the endogenous CHO cell integrin, αβ12 (30). Expression of P(Ala176–Lys277) or of P(Met1–Lys125) had minimal, statistically insignificant effects on cell migration on FN (Fig. 7, lower panel). In addition, expression of these fragments did not affect cell adhesion to either fibronectin or FN (data not shown). Thus, introduction of an inhibitor of the paxillin-α4 interactions selectively blocked α4 tail-mediated cell migration.

To further analyze the effect of P(Ala176–Lys277) on α4-dependent cell migration, we performed cell migration assays using time lapse video microscopy. Cells expressing P(Ala176–Lys277) were significantly less motile on fibronectin, 5.5 ± 0.6 mm/h, than those expressing vector control, 12.5 ± 2.6 mm/h, or untransfected cells, 14.2 ± 2.6 mm/h (Fig. 8, A and C). In contrast, cells expressing this fragment migrated at a significantly higher rate, 9.4 ± 0.9 mm/h, on FN (Fig. 8, B and C). Thus, P(Ala176–Lys277) specifically inhibited the α4 tail-dependent cell random migration.

**DISCUSSION**

In the current study, we have mapped the α4 integrin-binding region within paxillin and examined its effect on the α4 cytoplasmic domain-mediated cellular functions. We found that: 1) sequences between residues Ala176 and Asp275 of paxillin are sufficient for binding to the α4 tail; 2) the α4 integrin tail, paxillin, and FAT can form a ternary complex; 3) the α4-binding paxillin fragment, Ala176–Asp275, specifically blocked paxillin binding to the α4 tail more efficiently than it blocked binding to FAT; and 4) this fragment specifically blocked cell migration stimulated by the α4 cytoplasmic domain. Thus, this fragment contains sequences that are required for binding to the α4 cytoplasmic domain and can function as a dominant negative inhibitor of α4 integrin-mediated cellular functions.

Previously, we suggested that the α4, paxillin, and pp125FAK might form a ternary complex. This complex might increase the membrane targeting and clustering of pp125FAK and induce the rapid phosphorylation of pp125FAK, which might account for the increased cell migration in the α4-mediated cell adhesion. In the current study, we have provided direct evidence indicating that indeed the α4 integrin tail, paxillin, and FAT, the focal adhesion targeting domain of pp125FAK which contains the paxillin-binding region, can form a ternary complex. FAT was unable to bind the α4 tail directly and also did not inhibit paxillin binding to the α4 and it can only interact with the α4 through paxillin. In addition, our data indicate that the intact α4 integrin, paxillin, and pp125FAK can also form a ternary complex in cells. Thus, these data further support the direct association between the α4 and paxillin and suggest that the α4 and pp125FAK might interact with paxillin through different sites. Thus, the ternary complex of the α4 integrin and paxillin and pp125FAK might account for the rapid tyrosine phosphorylation and activation of pp125FAK. This increased activation of pp125FAK may then contribute to increased α4 integrin-mediated (10) cell migration because pp125FAK has been implicated in stimulating cell migration (23, 24).

The N-terminal domain of paxillin contains five LD motifs that mediate protein-protein interactions (12–14). For example, the LD1, LD2, and LD4 motifs mediate paxillin binding to vinculin, the LD2 and LD4 motifs are involved in its interaction with pp125FAK, and paxillin binds PKL through its LD4 motif (12, 13, 20). Since P(Ala176–Asp275), the α4-binding fragment identified in the current study contains both LD3 and LD4 interactions (12, 13, 20), we reasoned that it was possible that P(Ala176–Asp275) might also interfere paxillin-pp125FAK interaction. However, our results indicate that the P(Ala176–Asp275) fragment only partially (30% of inhibition at 100-fold molar excess, Fig. 6A) blocked paxillin binding to FAT, whereas the full-length paxillin effectively (>90% inhibition at 6-fold molar excess, Fig. 6B) blocked the binding. In sharp contrast, the same fragment effectively inhibited paxillin binding to the α4 tail, with 95% inhibition at a 20-fold molar excess. These data indicate that the P(Ala176–Asp275) fragment is more potent in inhibiting the α4-paxillin interaction than pp125FAK-paxillin interaction. One possible explanation is that since LD2 and LD4 motifs both can mediate pp125FAK-paxillin interaction (20), it is possible that in the presence of excess P(Ala176–Asp277) fragment, the pp125FAK-paxillin interaction is most likely mediated by the LD2 motif. Therefore, pp125FAK can still bind paxillin even though the P(Ala176–Asp277) fragment might affect pp125FAK-LD4 interaction.
Using two independent cell migration assays, that is, random cell migration using Transwell chambers and real-time video phase-contrast microscopy, we have shown that the α4-binding fragment of paxillin, P(Ala\textsuperscript{176}-Lys\textsuperscript{277}), when expressed in the α\textsubscript{4}β\textsubscript{α}β\textsubscript{1}A4-expressing CHO cells, effectively blocked the α4 tail-stimulated cell migration on fibrinogen, whereas it had a minor effect on cell migration on FN, a ligand for the endogenous α\textsubscript{4}β\textsubscript{1} integrin (Figs. 7 and 8). In contrast, P(Met\textsuperscript{1}-Lys\textsuperscript{125}), a paxillin fragment that failed to bind the α4 integrin-paxillin interaction and specifically inhibit α4-mediated cell migration (Figs. 7 and 8). In contrast, P(Met\textsuperscript{1-Lys\textsuperscript{125}}), a paxillin fragment that failed to bind the α4 integrin-paxillin interaction and specifically inhibit α4-mediated cell migration on fibrinogen, whereas it had no inhibitory effect on either α4 or α5-mediated cell migration (Fig. 7). Since pp125FAK-paxillin interaction may not have an inhibitory effect on either α4 or α5 integrins, we have shown that the α4 integrin-paxillin interaction is consistent with the modest effects in vitro reported here. Thus, this α4-binding paxillin fragment can function as a dominant negative effector for the α4 integrin-paxillin interaction and specifically inhibit the functions of α4 integrins.

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