Paxillin association in vitro with integrin cytoplasmic domain peptides

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Abstract Short cytoplasmic domains of integrin heterodimers are crucial for transduction of signals generated by adhesion of cells to the extracellular matrix. Here, we describe the use of peptides mimicking the intracellular tails of integrin $\alpha_5\beta_1$ to assay in vitro associations with cytoskeletal proteins. Our results suggest that the focal adhesion protein, paxillin, may interact directly with the intracellular region of the integrin β_1 subunit. Paxillin is known to form stable complexes with several signaling molecules, including focal adhesion kinase. Physical interaction between paxillin and the β_1 cytoplasmic domain suggests a model in which paxillin may function as a key intermediary in integrin-mediated signal transduction.

Key words: Integrin; Paxillin; Cell adhesion

1. Introduction

Cells adhere to the extracellular matrix (ECM) through heterodimeric ($\alpha\beta$) transmembrane receptors of the integrin family [1,2]. Integrin extracellular domains contact the ECM at specialized structures known as focal adhesion plaques [3,4], in which the intracellular domains of integrins provide nucleation sites for the assembly of protein complexes that bind actin filaments [5,6]. In addition to providing structural connections between the ECM and the actin cytoskeleton, integrin receptor stimulation generates intracellular signals [7–9] which may contribute to the regulation of anchorage-dependent cell growth, including tyrosine phosphorylation of a number of cellular proteins [10,11]. Since integrin cytoplasmic regions are short and lack intrinsic enzymatic activity, integrin-mediated signal transduction must rely on the recruitment and activation of signaling molecules appropriately positioned in proximity to integrin intracellular domains. Notably, plating of cells on fibronectin [12,13] or clustering of β_1 integrins by antibody cross-linking [14] results in tyrosine phosphorylation of a non-receptor protein tyrosine kinase (PTK), FAK, that localizes to focal adhesions [15–21]. FAK is capable of forming signaling complexes with several proteins including paxillin [22–24], a focal adhesion protein found in association with vinculin [25] that is multiply phosphorylated following integrin receptor engagement [26–31].

Though integrin cytoplasmic domains are apparently critical for the transduction of regulatory signals accompanying cell adhesion [32,33], the immediate consequences of integrin occupancy and the mechanism of FAK activation remain poorly understood. Much effort has focused on the identification of proteins that may interact with integrin cytoplasmic tails. In vitro binding assays using synthetic peptides based on the cytoplasmic regions of integrin subunits have elucidated structural features of focal adhesion complexes. Integrin β_1 peptides have been shown to bind to the actin-associated protein, α-actinin [34,35] and to talin [36,37]. Talin provides an indirect linkage to actin filaments through complexes with vinculin [38], which in turn, associates with α-actinin [39,40] and another actin-binding protein, tensin [41]. A recent study reported direct binding of FAK to β_1 cytoplasmic peptides, utilizing β_1 sequences distinct from those important for talin or α-actinin recognition [42]. Evidence was also presented for association of paxillin with β_1 in extracts of chicken embryo (CE) fibroblasts; however, binding of both FAK and paxillin was apparently mediated by the same membrane proximal region of the β_1 subunit [42], and the known physical interaction between FAK and paxillin [22–24] precluded resolution of whether paxillin associated directly or indirectly with integrin.

Here, we use peptides that mimic intracellular domains of the human fibronectin receptor ($\alpha_5\beta_1$) in binding assays to explore the role of paxillin in integrin-mediated signaling. We present new evidence that paxillin may interact directly with the cytoplasmic tail of the β_1 subunit. These results suggest a model in which paxillin may act as an important intermediary in transduction of signals generated by cell adhesion through integrins.

2. Materials and methods

2.1. Materials

Immunoblotting was performed using the following monoclonal antibodies: anti-paxillin (Zymed Laboratories, South San Francisco, CA); anti-FAK and anti-ezrin (Transduction Laboratories, Lexington, KY); anti-talin, anti-α-actinin, anti-vinculin (Sigma Immunochemicals, St. Louis, MO); anti-tensin (Chemicon International, Temecula, CA); anti-FLAG M2 monoclonal antibody (Eastman Kodak Co., New Haven, CT) and polyclonal anti-human β_1 integrin cytoplasmic domain (Chemicon International). Protein tyrosine phosphorylation was examined with anti-phosphotyrosine antibody RC20 (Transduction Laboratories).

2.2. Peptide synthesis

Peptides corresponding to human integrin α_5 and β_1 cytoplasmic tails were synthesized by standard protocols using Fmoc chemistry on an Applied Biosystems peptide synthesizer, Model 430A (Foster City, CA). Amino acid sequences of peptides were confirmed using an Applied Biosystems Model 477A sequencer. The procedure for cross-

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linking of α_5 and β_1 peptides to a biotinylated linker will be reported elsewhere (Tanaka et al., in preparation). The M_r of the $\alpha_5\beta_1$ peptide construct was verified by mass spectrometry; m/z=9748.46, calculated for $C_{446}H_{690}O_{121}N_{112}S_6$=9749.46.

2.3. Cell culture and preparation of extracts

KB cells were obtained from the Japanese Cancer Research Resources Bank and were cultured in DMEM supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Adhesion of KB cells to ECM proteins was examined using Cytomatrix Cell Adhesion Strips (Chemicon International) according to the supplier's specifications. Adherent cells stained with 0.2% crystal violet were quantitated by measuring absorbance at 540 nm. To prepare cell extracts, KB monolayers (approx. 1×10^7 cells) plated on 100-mm dishes coated with fibronectin at 10 μg/ml were washed twice with phosphate-buffered saline (PBS) containing 500 μM Na_3VO_4. For peptide binding assays, cells were lysed for 15 min at 4°C in 1% Nonidet P-40 buffer (10 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin, pepstatin, and leupeptin). Insoluble material was removed by centrifugation at 14000 rpm for 10 min. Suspended cells were detached from tissue culture dishes by treatment with 2 mM EDTA in PBS at 37°C for 15 min, harvested by centrifugation, and then lysed in 1% Nonidet P-40 buffer. For immunoprecipitations with monoclonal antibodies, adherent or detached cells were lysed in modified RIPA buffer essentially as described [43]. Pre-cleared lysates were incubated with monoclonal antibodies for 2 h at 4°C and immune complexes precipitated using protein G-Sepharose beads (Pharmacia, Piscataway, NJ).

2.4. Peptide binding assays

0.5 ml of total KB cell lysates (500 μg) in 1% Nonidet P-40 buffer were rotated for 3 h at 4°C with 20–500 ng of biotin-labeled $\alpha_5\beta_1$ or β_1 peptide conjugated to Dynabeads M-280 Streptavidin (Dynal AS, Oslo, Norway). Non-specific binding interactions were blocked by pre-incubation of the peptide-conjugated beads in PBS containing 0.1% bovine serum albumin (BSA) for 1 h at room temperature. Cellular proteins complexed with paramagnetic beads were collected using a Dynal MPC-E magnetic particle concentrator (Dynal AS). Beads were washed five times with 1% Nonidet P-40 buffer prior to elution with SDS sample buffer. Some incubations were carried out in the presence of 2 μl of polyclonal antiserum generated against the human β_1 integrin peptide. Incubations of cell lysates with paramagnetic beads lacking the $\alpha_5\beta_1$ cross-linked peptide were included as negative controls in all experiments. Proteins bound to $\alpha_5\beta_1$ beads were identified by immunoblotting with antibodies recognizing focal adhesion proteins. Bead complexes were analyzed by SDS-PAGE and immunoblotting as described [43]. Visualization of proteins on immunoblots was carried out using enhanced chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL). Binding of purified recombinant GST-paxillin to approx. 10 ng of β_1 or control paramagnetic beads was assayed in 0.5 ml of PBS containing 1% Nonidet-P40 using approx. 5 ng of glutathione-*S*-transferase (GST)-paxillin fusion protein or purified GST control. After extensive washing of beads in PBS 1% Nonidet-P40, proteins were eluted with SDS sample buffer. GST-paxillin binding was assessed by silver staining of SDS gels and by immunoblotting with anti-paxillin antibody.

2.5. Protein expression in E. coli

Human paxillin was produced in *E. coli* as a GST fusion using vector pGEX-2T (Pharmacia), containing the entire coding region of human paxillin cDNA (Y. Masaki, S. Hashimoto and H.S., manuscript in preparation) isolated from U937 cells based on the published sequence [30]. The 94 kDa GST-paxillin fusion protein was purified using glutathione-Sepharose beads as described (Pharmacia). Human FAK cDNA [44] was provided by Dr. S.B. Kanner (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). A C-terminal fragment of FAK (Q852-H1052) comprising the focal adhesion targeting (FAT) domain [22–24] was expressed in *E. coli* as a FLAG epitope-tagged fusion and purified using anti-FLAG M2 affinity matrix (Eastman Kodak Co.). Paxillin was co-immunoprecipitated from KB cell extracts (200 μg) prepared in 1% Triton X-100, 150 mM NaCl, 10 mM Tris-Cl pH 7.4, 1 mM EDTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5% Nonidet P-40 buffer using 2 μg of purified epitope-tagged FAT protein and anti-FLAG M2 antibody.

3. Results

3.1. Adhesive properties of human KB cells and adhesion-dependent tyrosine phosphorylation of paxillin

Interaction of paxillin or other focal adhesion proteins with fibronectin receptor ($\alpha_5\beta_1$) cytoplasmic domains was investigated in human KB carcinoma cells. As depicted in Fig. 1A, KB cells adhere efficiently to tissue culture wells coated with the ECM proteins fibronectin, laminin, collagen type IV, and tenascin, consistent with reported expression of α_2, α_3, α_5, α_6, and β_1 integrin subunits at the cell surface [10]. When we compared protein tyrosine phosphorylation patterns in extracts of KB cells spread on fibronectin-coated dishes with rounded KB cells that were detached from tissue culture dishes and kept in suspension, several phosphotyrosine-containing proteins were significantly enriched in adherent cells (Fig. 1B,a, compare lanes 1,2). Immunoprecipitation experiments identified 125 and 68 kDa phosphoproteins as FAK ([10,14], data not shown) and paxillin (Fig. 1B,c), respectively. Extracts of cells plated on fibronectin showed enhanced tyrosine phosphorylation of paxillin in immunoprecipitates relative to cells kept in suspension (Fig. 1B,b, lanes 1,2), establishing adhesion-dependent regulation of paxillin tyrosine phosphorylation in KB cells. These data are in good agreement with findings in other cell types implicating paxillin as a downstream component in integrin-mediated signaling pathways [26–31].

3.2. Design of a heterodimeric peptide mimicking $\alpha_5\beta_1$ cytoplasmic domains

To assay protein association with intracellular domains of the $\alpha_5\beta_1$ fibronectin receptor in KB cells, we synthesized a heterodimeric peptide composed of sequences identical to the cytoplasmic regions of the human α_5 and β_1 subunits (Fig. 2). α_5 and β_1 peptides were joined to a short, biotin-conjugated peptide linker at their amino-termini using a peptide architecture technique outlined in Section 2. Incorporation of biotin into the linker facilitated use of the integrin receptor mimic, in conjunction with streptavidin-conjugated paramagnetic beads, to affinity-purify cellular proteins bound to $\alpha_5\beta_1$ cytoplasmic tails. Homodimeric ($\alpha_5\alpha_5$ or $\beta_1\beta_1$) cytoplasmic domain peptides were also generated and linked to paramagnetic beads by similar procedures for use in binding studies (not shown).

3.3. Paxillin binding to $\alpha_5\beta_1$ beads in extracts of adherent KB cells

Fig. 3a shows immunoblot analyses of $\alpha_5\beta_1$ peptide-associated proteins purified from extracts of KB cells adhering to fibronectin. As expected, proteins bound to $\alpha_5\beta_1$ cytoplasmic domain peptides included two proteins, talin and α-actinin, known from previous in vitro studies to interact with the β_1 intracellular region [34–37]. Isolation of talin and α-actinin confirmed the utility of the heterodimeric peptide in identification of proteins that interact with the $\alpha_5\beta_1$ cytoplasmic domain. α-Actinin bound particularly well to the $\alpha_5\beta_1$ peptide (Fig. 3a). Incubation with peptide-coated beads produced a significant enrichment of α-actinin over marginal protein levels detected in total cell lysates (TCL), relative to other focal adhesion proteins, and α-actinin binding persisted using a quantity of beads 25-fold lower (Fig. 3a, lane 4) than that at which binding of talin was detected (Fig. 3a, lane 2). Other

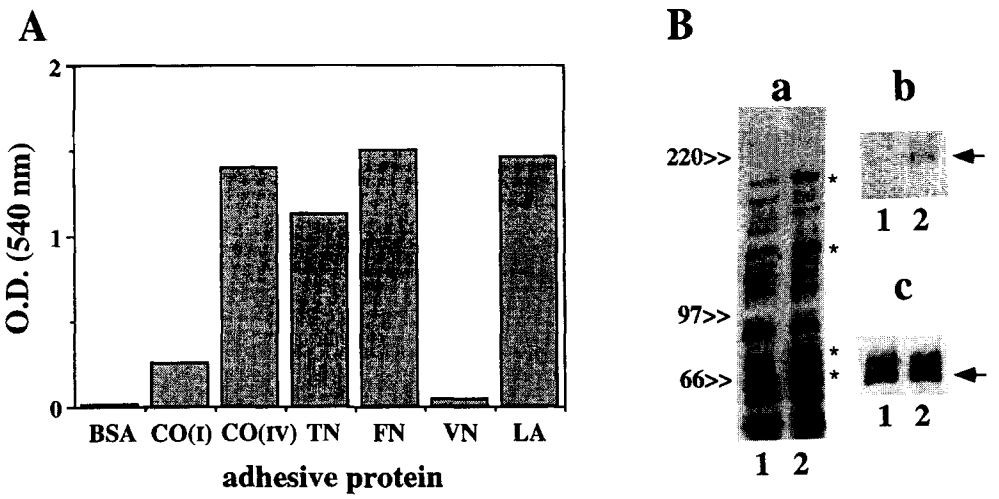

Fig. 1. Enhanced tyrosine phosphorylation of paxillin in adherent KB cells. (A) Adhesion of KB cells to various ECM substrates was examined as described in Section 2. Each data point represents the mean of 10 replicate microtiter wells coated with adhesive protein. BSA, bovine serum albumin; CO(I), collagen type I; CO(IV), collagen type IV; TN, tenascin; FN, fibronectin; VN, vitronectin; LA, laminin. (B) Phosphotyrosine-containing cellular proteins in total cell lysates of suspended (a, lane 1) or fibronectin-plated (a, lane 2) KB cells were examined by immunoblotting with anti-phosphotyrosine antibody. Molecular masses of marker proteins are indicated in kD. Asterisks denote cellular proteins which show enhanced phosphorylation on tyrosine in adherent relative to suspended cells. (b) Immunoprecipitation of paxillin from lysates of suspended (lane 1) or fibronectin-plated (lane 2) KB cells was performed using anti-paxillin followed by immunoblot analysis with anti-phosphotyrosine. (c) Immunoblotting with anti-paxillin confirmed equivalent levels of paxillin in immunoprecipitates from suspended (lane 1) or adherent (lane 2) KB cells. Arrows indicate paxillin.

focal adhesion proteins associated with $\alpha_5\beta_1$ beads in in vitro assays were vinculin, and paxillin (Fig. 3a). Neither of these proteins has been implicated previously in direct interactions with integrin subunits. Rather, in vitro data suggest that vinculin associates with integrin indirectly, through complexes with talin [38] or α-actinin [39]; while paxillin is known to bind vinculin [25]. Under conditions in which binding of KB cellular α-actinin, talin, vinculin, and paxillin to $\alpha_5\beta_1$ beads was readily detected, no significant associations with endogenous FAK, tensin (Fig. 3a), β-actin or ezrin (data not shown) were observed.

3.4. *Paxillin binding to $\alpha_5\beta_1$ beads in extracts of suspended KB cells*

To examine further the binding of paxillin and other focal adhesion proteins to $\alpha_5\beta_1$ cytoplasmic domains, we repeated peptide binding assays using extracts of suspended KB cells (Fig. 3b–d). By disruption of focal contacts, we hoped to gain insight into potentially direct physical interactions of focal adhesion proteins with $\alpha_5\beta_1$ peptides as opposed to indirect interactions dependent upon the presence of existing protein complexes in lysates of cells spread on fibronectin. Significantly, binding of cellular paxillin (Fig. 3b, lane 2), talin and α-actinin (Fig. 3c, lane 2), but not vinculin (Fig. 3b, lane 2), to $\alpha_5\beta_1$ beads was detected in extracts of suspended KB cells. In addition, interaction of paxillin with $\alpha_5\beta_1$ cytoplasmic domain peptides was inhibited in the presence of antiserum generated against a peptide derived from the human β_1 intracellular subunit (Fig. 3b, lane 3). Further studies using extracts of suspended cells and beads coated with either homodimeric α_5 or β_1 peptides (Fig. 3d) provided evidence for selective interaction of paxillin with the β_1 (Fig. 3d, lane 4), but not the α_5 (Fig. 3d, lane 3) cytoplasmic tail.

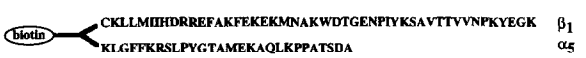

Fig. 2. Heterodimeric peptide mimicking human fibronectin receptor α_5 and β_1 cytoplasmic domains. Peptides corresponding to integrin α_5 and β_1 intracellular regions were synthesized separately and their amino-terminal regions were coupled to a peptide linker as summarized in Section 2. The amino-terminal lysines of α_5 and β_1 cytoplasmic peptides are presumed to represent the residues just proximal to the interface between membrane and cytoplasm [6]. Cysteine was included at the extreme amino-terminus of β_1 for peptide coupling. The biotin moiety conjugated to the linker permits isolation of peptide-protein complexes. Biotin is at a position distinct from the α_5 and β_1 sequences in our synthetic scheme to reduce steric hindrance in binding of intracellular ligands.

3.5. *Binding of GST-paxillin to the β_1 cytosplamic domain*

In vitro studies with purified recombinant paxillin were performed to establish whether paxillin binding to β_1 may occur directly (Fig. 4). Full-length human paxillin was expressed in *E. coli* as a GST fusion protein, and purified GST-paxillin was incubated with β_1-coated or uncoated control beads as described in Section 2. Bead complexes were resolved by SDS-PAGE and analyzed by immunoblotting with anti-paxillin (Fig. 4a) or by silver staining (Fig. 4b). Immunoblotting experiments detected apparent physical association of GST-paxillin with β_1 beads (Fig. 4a, lane 3). Significant levels of non-specific binding of GST-paxillin to uncoated beads were not observed (Fig. 4a, lane 2). Silver staining of duplicate SDS gels was consistent with direct and specific interaction of GST-paxillin with the β_1 cytoplasmic peptide (Fig. 4b, compare lanes 2,3). Importantly, no binding of purified GST to β_1 was detected (Fig. 4b, lane 1), indicating that interaction of GST-paxillin with the β_1 cytoplasmic tail is unlikely to depend on the GST moiety.

3.6. *KB cellular paxillin associates with the C-terminal domain of FAK*

Studies in other cell types have established formation of a signaling complex composed of FAK and paxillin [22–24],

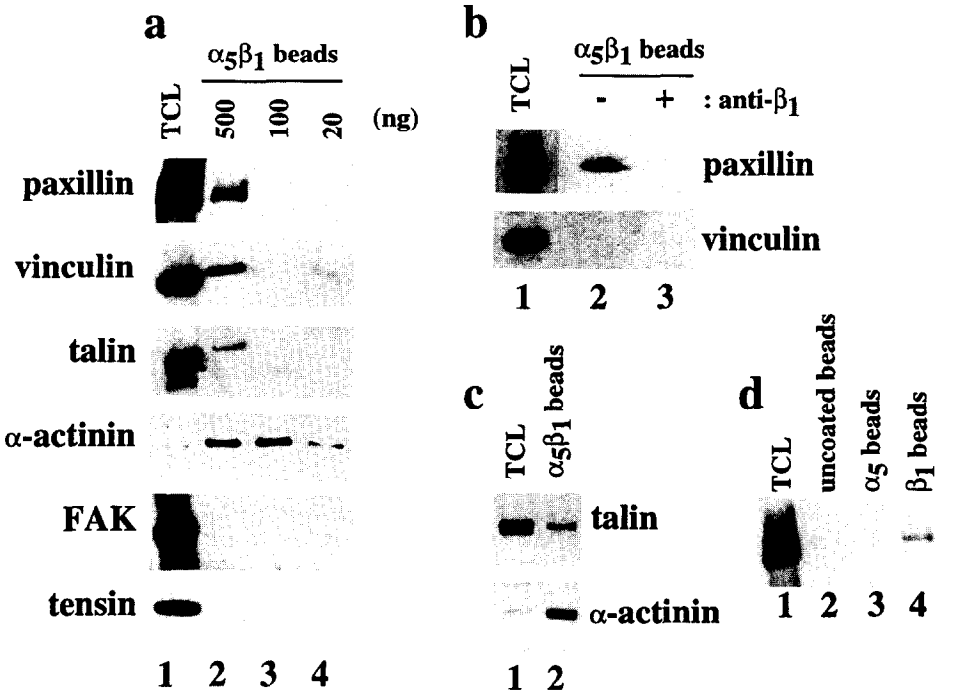

Fig. 3. Paxillin from lysates of adherent or suspended KB cells binds to $\alpha_5\beta_1$ beads. Total cell lysates (TCL, lane 1) from fibronectin-plated or suspended KB cells were incubated with $\alpha_5\beta_1$ beads as described in Section 2. Proteins bound to beads were identified by immunoblotting with monoclonal antibodies recognizing the indicated focal adhesion proteins. Extracts of adherent KB cells (a) were incubated with 500 (lane 2), 100 (lane 3) or 20 (lane 4) ng of $\alpha_5\beta_1$ peptide-coated beads. Extracts of suspended KB cells (b,c) were incubated with 500 ng of $\alpha_5\beta_1$ beads (lane 2) or 500 ng of $\alpha_5\beta_1$ beads plus antiserum against the human β_1 cytoplasmic peptide (b, lane 3). (d) Incubation of suspended KB cell extracts with uncoated (lane 2), α_5 peptide-coated (lane 3), or β_1 peptide-coated (lane 4) beads. The data shown are from duplicate filters or, in some cases, from stripping and reprobing of the same filter with a different monoclonal antibody.

mediated by sequences comprising the C-terminal focal adhesion targeting (FAT) domain of FAK. Co-immunoprecipitation experiments using a purified C-terminal fragment of FAK confirmed that KB cellular paxillin is indeed competent to bind FAK in vitro (Fig. 5). Incubation of KB cell lysates with a FLAG epitope-tagged portion of FAK containing the paxillin-binding and FAT region [22–24] resulted in efficient immunoprecipitation of endogenous paxillin by FLAG M2 antibody (Fig. 5, lane 1). Thus, paxillin association with the β_1 cytoplasmic tail could conceivably promote cellular targeting of both paxillin and FAK to focal adhesions in KB cells.

4. Discussion

Our results suggest that paxillin may bind directly to the intracellular region of integrin $\alpha_5\beta_1$. Like two focal adhesion proteins known from in vitro studies to associate directly with the integrin β_1 cytoplasmic subunit, talin and α-actinin [34–37], paxillin from adherent KB cell extracts associated readily with $\alpha_5\beta_1$ or β_1 peptide-coated beads. Although we cannot entirely rule out the possibility that binding of paxillin to β_1 beads might be mediated by another protein that could be associated with paxillin, two lines of evidence suggest that paxillin may interact directly with the β_1 cytoplasmic domain. First, binding of paxillin to $\alpha_5\beta_1$ or β_1 beads was still detected in extracts of KB cells kept in suspension to disrupt preformed focal adhesion complexes. In contrast, vinculin, which is known to form complexes with both talin and paxillin [24,38], was found in association with $\alpha_5\beta_1$ beads only from extracts of KB cells spread on fibronectin. Second, we obtained in vitro evidence consistent with a direct physical interaction between purified recombinant GST-paxillin and the β_1 cytoplasmic domain.

In addition to its integrin-binding activity, co-immunoprecipitation experiments using an epitope-tagged C-terminal FAT domain of FAK indicated that KB cellular paxillin is competent to bind FAK. This finding is in good agreement with earlier studies showing that paxillin forms a stable complex with FAK [22] in adherent or suspended CE fibroblasts [23], as well as in a T cell line [24] utilizing sequences comprising the FAK C-terminal FAT domain. Thus, direct physical association of paxillin with the integrin β_1 intracellular subunit may be an important factor in localization of FAK, as well as paxillin itself, to focal adhesion sites. Recently, Schaller et al. [42] reported direct binding of FAK to peptides based on the integrin β_1 cytoplasmic subunit in lysates of CE cells engineered to over-express FAK. This interaction is likely to have important implications in FAK activation, but is unlikely to be responsible for targeting of FAK to focal adhesions as integrin binding is apparently mediated by sequences at the FAK N-terminus [42]. In the present work, we were not able to detect significant levels of association of endogenous KB cellular FAK with $\alpha_5\beta_1$ beads or β_1 beads (data not shown) under conditions where binding of cellular paxillin was easily detected, supporting the view that paxillin binding to β_1 occurs independently of FAK. Recent availability of a FAK-deficient mouse [45] will make it possible to ascertain whether paxillin localizes appropriately in vivo to focal contacts in the absence of FAK. Inability to detect FAK association with $\alpha_5\beta_1$ beads in our experiments could reflect prohibitively low protein levels of endogenous FAK in

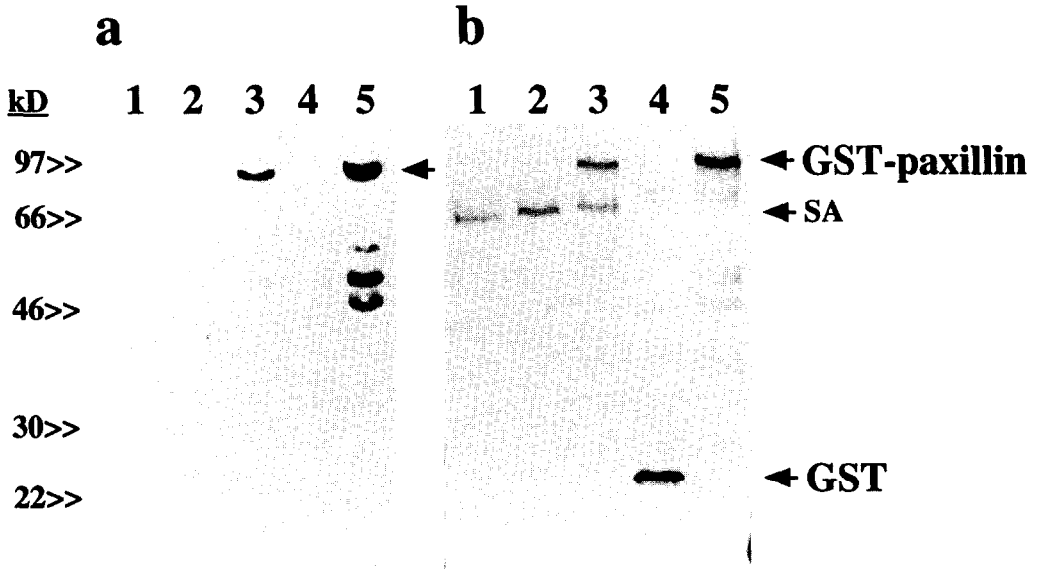

Fig. 4. Interaction of purified GST-paxillin with the β_1 cytoplasmic domain peptide. GST-paxillin or GST were expressed in *E. coli* and purified using glutathione-Sepharose. Binding to β_1 beads was assayed as described in Section 2. Bead complexes were resolved by SDS-PAGE and analyzed by immunoblotting with anti-paxillin antibody (a) or silver staining (b). β_1 peptide-coated beads were incubated with 5 ng of either GST (lane 1) or GST-paxillin (lane 3), while in lane 2, control uncoated beads were incubated with GST-paxillin. Purified preparations of GST and GST-paxillin (10 ng each) are shown in lanes 4 and 5, respectively. Molecular masses of marker proteins are indicated in kDa. The 60 kDa protein visible in lanes 1–3 of (b) is streptavidin (SA) originating from Dynabeads M-280.

KB cells compared to the system utilized by Schaller et al. [42] or low affinity of the FAK-integrin interaction.

Emerging evidence supports the view that integrin cytoplasmic regions act not only as templates for the formation of focal adhesion structures, but also in the recruitment of PTKs and other proteins with signaling function, including paxillin. It is attractive to speculate that one role of paxillin and, possibly, of talin which like paxillin forms complexes with both the integrin β1 intracellular domain [36,37] and FAK [46], might be to help position cellular PTKs in proximity to integrin cytoplasmic domains, thereby facilitating their activation in response to integrin receptor occupancy. Thus, despite their small size and limited amino acid sequence information, integrin intracellular domains appear to function as versatile signal transducers through multiple associations with regulatory components of focal adhesion networks.

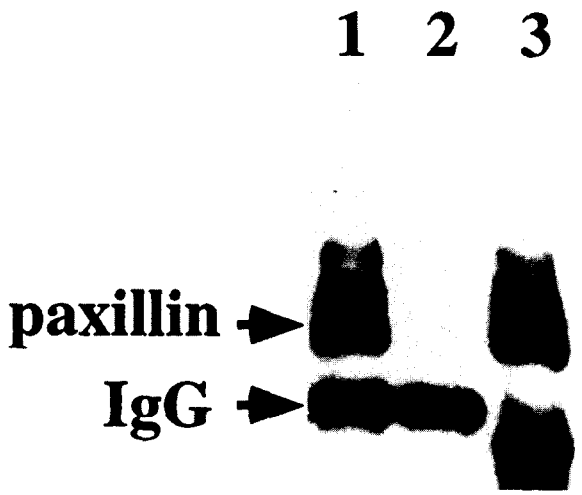

Fig. 5. KB cellular paxillin associates with FAK. A C-terminal fragment of FAK including the FAT domain was expressed in *E. coli* as a FLAG epitope-tagged fusion and immunoaffinity purified as described in Section 2. Total cell lysates (200 μg) from adherent KB cells were incubated with 2 μg (lane 1) or 0 μg (lane 2) of purified FAT protein followed by immunoprecipitation with anti-FLAG M2 antibody. Lane 3 shows a control immunoprecipitation from 200 μg of KB total cell lysate using anti-paxillin antibody. Immunoprecipitates were resolved by immunoblotting with anti-paxillin.

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