Paxillin LD4 Motif Binds PAK and PIX through a Novel 95-kD AnkyrinRepeat, ARF–GAP Protein: A Role in Cytoskeletal Remodeling

Christopher E. Turner,* Michael C. Brown,* Joseph A. Perrotta,* M.C. Riedy,* Sotiris N. Nikolopoulos,* A. Rosa McDonald,* Shubha Bagrodia,† Sheila Thomas,§ and Phillip S. Leventhal*

*Department of Anatomy and Cell Biology, State University of New York, Health Science Center, Syracuse, New York 13210; †Department of Molecular Medicine, Cornell University, Ithaca, New York 14853; and §Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Abstract. Paxillin is a focal adhesion adaptor protein involved in the integration of growth factor- and adhesion-mediated signal transduction pathways. Repeats of a leucine-rich sequence named paxillin LD motifs (Brown M. C., M. S. Curtis, and C. E. Turner. 1998. Nature Struct. Biol. 5:677–678) have been implicated in paxillin binding to focal adhesion kinase (FAK) and vinculin. Here we demonstrate that the individual paxillin LD motifs function as discrete and selective protein binding interfaces. A novel scaffolding function is described for paxillin LD4 in the binding of a complex of proteins containing active p21 GTPase–activated kinase (PAK), Nck, and the guanine nucleotide exchange factor, PIX. The association of this complex with paxillin is mediated by a new 95-kD protein, p95PKL (paxillin-kinase linker), which binds directly to paxillin LD4 and PIX. This protein complex also binds to Hic-5, suggesting a conservation of LD function across the paxillin superfamily. Cloning of p95PKL revealed a multidomain protein containing an NH₂-terminal ARF–GAP domain, three ankyrin-like repeats, a potential calcium-binding EF hand, calmodulin-binding IQ motifs, a myosin homology domain, and two paxillin-binding subdomains (PBS). Green fluorescent protein–(GFP-)tagged p95PKL localized to focal adhesions/complexes in CHO.K1 cells. Overexpression in neuroblastoma cells of a paxillin LD4 deletion mutant inhibited lamellipodia formation in response to insulin-like growth factor-1. Microinjection of GST–LD4 into NIH3T3 cells significantly decreased cell migration into a wound. These data implicate paxillin as a mediator of p21 GTPase–regulated actin cytoskeletal reorganization through the recruitment to nascent focal adhesion structures of an active PAK/PIX complex potentially via interactions with p95PKL.

Key words: migration • signal transduction • integrins • trafficking • vesicular transport

The Rho family of p21 GTPases plays a pivotal role in regulating changes in the actin-based cytoskeleton observed in response to growth factors and cell adhesion. A hierarchy is suggested in which activation of Cdc42 progresses through Rac to Rho (Ridley and Hall, 1992; Hall, 1994). Rho A has been implicated in the formation of focal adhesions and actin stress fibers, while Cdc42 and Rac activation stimulate the more dynamic processes of filopodial and lamellipodial extension, respectively, in association with focal complex formation (Nobes and Hall, 1995). The precise mechanism(s) by which these GTPases remodel the actin cytoskeleton remains to be determined, although direct association of serine/threonine kinases with the individual GTPase is likely a primary mediator of these effects. For example, Rho-associated protein kinase N and Rho kinase increase the activity of myosin light chain kinase (MLCK)1 and myosin, triggering the generation of tension along the actin and myosin-containing stress fibers, leading to focal adhesion formation (Burdridge and Chrzanowska-Wodnicka, 1996; Leung et al., 1996; Narumiya, 1996). Conversely, p21 GTPase–activated kinase (PAK) activity has been linked to focal adhesion disassembly (Manser et al., 1997), possibly through its ability to phosphorylate and thereby inhibit MLCK (Sanders et al., 1999). PAK, a serine/threonine kinase, binds tightly...
to activated Cdc42 and Rac1, and has been localized to sites of primary cell–matrix interaction called focal complexes (Nobes and Hall, 1995; Lim et al., 1996). However, the role of PAK in Cdc42- and Rae-mediated filopodia and lamellipodia formation is unclear (Nobes and Hall, 1995; Lamarche et al., 1996; Manser et al., 1997; Sells et al., 1997; Westwick et al., 1997).

The focal complexes and focal adhesions formed in response to p21 GTPase activity are sites of integrin engagement with the extracellular matrix that consist of structural proteins, such as talin and vinculin, that link integrins to the actin cytoskeleton and regulatory proteins, such as FAK and Src, that likely transduce integrin-, as well as growth factor-dependent signals (Clark and Brugge, 1995; Craig and Johnson, 1996). Paxillin likely serves a multidomain adaptor function at these sites (Turner et al., 1990; Tachibana et al., 1995; Turner, 1998). Indeed, the paxillin NH2 terminus contains binding sites for the nonreceptor kinases FAK, PYK2, Csk, Src, and the structural protein vinculin (Sabe et al., 1994; Turner and Miller, 1994; Schaller and Parsons, 1995; Tachibana et al., 1995; Salgia et al., 1995), whereas the COOH terminus, composed of four LIM domains, mediates paxillin targeting to focal adhesions (Brown et al., 1996, 1998a).

Paxillin has been implicated in the regulation of focal adhesion assembly and signal transduction through these elements, primarily as a result of its propensity to become tyrosine phosphorylated in response to cell adhesion to extracellular matrix and following challenge of serum-starved cells with soluble growth factors (Burridge et al., 1998a). Paxillin likely serves a multidomain adaptor function at these sites (Turner et al., 1990; Tachibana et al., 1995; Turner, 1998). Indeed, the paxillin NH2 terminus contains binding sites for the nonreceptor kinases FAK, PYK2, Csk, Src, and the structural protein vinculin (Sabe et al., 1994; Turner and Miller, 1994; Schaller and Parsons, 1995; Tachibana et al., 1995; Salgia et al., 1995), whereas the COOH terminus, composed of four LIM domains, mediates paxillin targeting to focal adhesions (Brown et al., 1996, 1998a).

We have identified a leucine-rich motif with the consensus LDXXLXXL that is repeated five times within the NH2 terminus of paxillin (Brown et al., 1996, 1998b). The presence of these motifs within the binding sites on paxillin for FAK, vinculin, and the E6 oncoprotein from papillomavirus (Tong et al., 1997; Vande Pol et al., 1998), as well as their occurrence in other diverse protein families, suggests that the LD motif represents a novel protein-binding module of general importance (Brown et al., 1998b).

In this report, we present evidence that the individual LD motifs of paxillin function as independent and selective protein-binding sites. Furthermore, we show that through the LD4 motif, paxillin binds a protein complex containing PAK, the adaptor protein Nck, two members of the Cdc42/Rac1 guanine nucleotide exchange factor (PIX/COOL/p85SPr family; Woo et al., 1997; Bagrodia et al., 1998; Manser et al., 1998), and a new multidomain ARF–GAP protein, p95PKL (paxillin-kinase linker; pronounced pickle). p95PKL binds paxillin and PI(4,5)P2 directly in vitro. The association of paxillin with this complex suggests a potential function for paxillin in recruiting these proteins to the plasma membrane and thereby directly mediating p21 GTPase reorganization of the actin-based cytoskeleton.

### Materials and Methods

#### Materials

PAK antibody was purchased from Santa Cruz, vinculin (Vin 11-5) from Sigma Chemical Co., and Nck, p130Cas, and clathrin heavy chain antibodies were obtained from Transduction Labs. FAK antibody (BC3) was a generous gift of Dr. J.T. Parsons (University of Virginia, Charlottesville, VA), antitalin (8d4) was provided by Dr. K. Burridge (University of North Carolina, Chapel Hill, NC), p50-COOL, and p130Cas, which recognizes p85/p75PIX/COOL, was as described (Bagrodia et al., 1998).

#### Preparation of Fusion Proteins and Binding Assays

Individual glutathione S-transferase (GST) fusion proteins spanning paxillin amino acid (aa) 54–313, or encompassing LD1 (aa 1–20), LD2 (aa 141–160), LD3 (aa 212–231), LD4 (aa 263–282), and LD5 (aa 299–317) were expressed in Escherichia coli (DH5α) and purified on glutathione agarose beads as previously described (Turner and Miller, 1994; Brown et al., 1996).

Tissue lysates (newborn rat brain, a gift of Qin He, State University of New York, Syracuse, NY, or embryonic day 17 chicken gizzard smooth muscle) were prepared by Dounce homogenization in 10 vol lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 0.1% β-mercaptoethanol, 0.5% TX-100) containing protease inhibitors (Complete™ EDTA-free; Boehringer Mannheim), followed by clarification for 15 min at 15,000 g. Alternatively, cultured cells were washed twice with ice-cold Hank’s buffer and processed as for tissue lysates. Lysates (0.5–1 mg protein) were incubated with the individual fusion proteins (50 μg) in fusion-protein-binding buffer (same as lysis buffer, but with the Triton X-100 adjusted to 0.1%) for 120 min at 4°C and then washed four times in binding buffer. The washed precipitates were boiled in SDS-PAGE sample buffer, followed by SDS-PAGE and Western blot analysis, or processed for in vitro kinase assay.

For in vivo coprecipitation experiments, rat brain was homogenized in 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1% NP-40, 10% glycerol. Paxillin was precipitated with antipaxillin antibody (Transduction Labs) and the blots probed with antibodies to PIX, PAK, paxillin, and p30Cas. Mouse IgG was used as a control. To demonstrate an association in vivo between paxillin and p95PKL, green fluorescent protein– (GFP–) p95PKL was transfected into COS 7 cells. 72 h after transfection, the cells were lysed in 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1% NP-40, 10% glycerol, and clarified by centrifugation at 15,000 g for 15 min. Paxillin was precipitated with antipaxillin antibody and the blots probed with antibodies to paxillin, GFP, and p130Cas. Mouse IgG was used in a control precipitation.

#### In Vitro Kinase Assays

Fusion protein precipitates were washed four times in fusion protein lysis buffer and once in kinase buffer (20 mM Tris-HCl, pH 7.6, 20 mM MgCl2, 10 mM MnCl2, 1 mM EDTA, 1 mM EGTA, 40 μM ATP). The pellets were resuspended in 20 μl kinase buffer and incubated for 20 min at room temperature in the presence of 5 μCi [32P]γ-ATP (>4,000 Ci/mmol; ICN Biomedicals). The effect of activated p21 GTPases on precipitated kinase activity was determined by the addition of GTPγS or GDP-loaded GST p21 GTPases (Rho, Rac, or Cdc42) and 2.5 μg myelin basic protein, as previously described (Bagrodia et al., 1995). The reactions were terminated by boiling in SDS-PAGE sample buffer. To evaluate if phosphorylation of precipitated proteins regulated binding to the paxillin fusion protein, the kinase reaction was followed (see Fig. 8) by several washes of the immobilized GST fusion protein complex with kinase buffer before boiling in SDS-PAGE sample buffer. Results were visualized by SDS-PAGE on 10 or 15% gels followed by autoradiography.

#### Metabolic Labeling

Asynchronously growing CHO.K1 fibroblasts were washed in serum-free DME and then incubated for 24 h in labeling buffer (8 ml methionine- and cysteine-free DME, 1 ml complete DME, 1 ml FBS [Summit Biotechnologies], 2 mM glutamine, 200 μCi Trans[35S]sulfetyl [>1,000 Ci/mmol; ICN Biomedicals]). The cells were washed in Hank's buffered saline and lysed in lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EGTA, 2 mM MgCl2, 0.1% β-mercaptoethanol, 0.5% Triton X-100) containing protease inhibitors. The lysate was clarified in a microfuge for 15 min at
Microsequencing and Cloning of p95PKL and p85PIX

LD4-binding proteins from chicken smooth muscle tissue lysate were separated by SDS-PAGE and transferred to PVDF membrane. Protein bands were visualized by Ponceau S staining and excised. Proteolytic digestion, HPLC fractionation, and internal peptide sequencing was performed by Dr. John Leszyk (Worcester Foundation for Biomedical Research, Worcester, MA).

Several of the peptide sequences generated from the p95 protein (p95PKL) precipitated by LD4 of paxillin were identical to the product of the KIAA0148 gene, which encodes a 50-kD protein (Nagase et al., 1995). The equivalent cDNA was isolated by reverse transcriptase-PCR from human U937 cells and a probe spanning nucleotides 527 to 999 of the KIAA0148 cDNA. Sequencing of a 2.5-kb clone revealed sequence in the 3′ end that coded for the additional peptide derived from the microsequencing of p95. A full-length clone of p95 was isolated and sequenced on the 5′ end. The sequence for p95PKL has been deposited in GenBank/EMBL/DDBJ accession number AF112366. Several additional p95PKL clones, representing alternative splice variants, were isolated and will be described elsewhere.

The cDNA for p85 PIX was generated by reverse transcriptase-PCR using RNA prepared from NIH3T3 cells. Forward and reverse primers were derived from the mouse PIX cDNA sequence (GenBank/EMBL/DDBJ U96634; Woo et al., 1997). p95PKL, p85PIX, and mPAK3 were derived from the mouse PIX cDNA sequence (GenBank/EMBL/DDBJ accession number U96634; Woo et al., 1997). The sequence for p95PKL has been deposited in GenBank/EMBL/DDBJ (Stratagene).

Results

Differential Binding of Vinculin and FAK to Paxillin LD Motifs

Paxillin comprises an NH2 terminus containing five LD motifs and a COOH terminus made up of four LIM domains (Fig. 1 A). Previously, we have localized the vinculin and FAK binding sites on the NH2 terminus of paxillin to extended regions encompassing LD motifs 2 and 4 (Brown et al., 1996). To determine the ability of vinculin and FAK to bind independently to the discrete paxillin LD motifs, each LD motif of paxillin was synthesized as a GST fusion protein (Fig. 1 C) and incubated with unlabelled smooth muscle cell lysate in precipitation-binding assays followed by Western blotting. Consistent with our previous findings (Brown et al., 1996), FAK was precipitated by LD4, and to a lesser extent, LD2 (Fig. 1 B). Vinculin exhibited strong binding to LD2, the domain previously identified as the principal binding site for this protein, in addition to a low binding to LD4. In contrast, FAK, vinculin also bound to LD1. Both vinculin and FAK binding to the aa 54–313 paxillin fusion protein was observed as previously shown (Brown et al., 1996). Thus, FAK and vinculin differentially associate with the individual paxillin LD motifs (Fig. 1 B). A reprobe of the blot confirmed that smooth muscle talin is unable to bind to the individual LD motifs or to aa 54–313 of paxillin (Fig. 1 B).

Individual Paxillin LD Motifs Exhibit Selective Protein Binding

Having established that the individual LD motifs can support specific and differential binding of known focal adhesion binding partners for paxillin, we determined if the individual LD fusion proteins associate with additional cellular proteins. GST–LD fusion proteins were incubated with [35S]methionine/cysteine metabolically labeled CHO.K1 lysates. GST–LD-binding proteins were visualized by SDS-PAGE followed by fluorography (Fig. 2). Each LD motif precipitated a discrete profile of cellular proteins. LD1 precipitated a protein of 120 kD (vinculin) and a prominent band of 50 kD. LD2 precipitated protein(s) of 120 kD (vinculin and FAK). LD3 and 5 precipitated no obvious 35S-labeled binding partners in this assay. In contrast, LD4 precipitated several prominent proteins ranging from 80 to 150 kD. Importantly, a paxillin fusion protein spanning aa 54–313, and therefore containing LD2–5, precipitated a complement of proteins similar to the individual LD motifs (Fig. 2). This suggests that the binding of proteins to the individual paxillin LD motifs

Cell Migration Assay

GST fusion proteins of paxillin LD4 were purified from bacterial lysates as described previously and dialyzed against microinjection buffer (10 mM KPO4, pH 7.5, 5 mM KCl, 0.1% β-mercaptoethanol). Aliquots of diazoyl fusion protein (1 mg/ml) were stored at −70°C. Microinjection was performed using standard protocols (Gilmore and Romer, 1996) using a Narishige micromanipulator and borosilicate glass capillary needles (World Precision Instruments) pulled on a Brown-Flaming micropipette puller (Sutter Instrument). To measure migration rates of injected cells, confluent NIH3T3 cells were wounded by clearing the cells from one side of the coverslip with a Teflon spatula. The first line of cells immediately adjacent to the wound were injected with either GST or GST–LD4. Cells were allowed to migrate for 24 h and then fixed and processed for immunofluorescence microscopy. Slides were coded, and microinjected cells scored blind for position in one of three categories: baseline, cells remaining at the edge of the original wound, i.e., at the site of injection; pack, cells which had migrated, but were not at the leading edge; and leading edge, cells at the front of the migrating pack. Four replicates were examined for each injected protein (>200 cells per treatment) and the data analyzed by r test.
was comparable to their binding when presented in the context of an intact NH$_2$ terminus. Similar results were obtained using lysates from NIH3T3 and Swiss 3T3 fibroblasts (data not shown). The 68-kD protein precipitated in approximately equal amounts by all fusion proteins also is precipitated to the same extent by GST alone (not shown), and is likely a heat shock protein.

Identification of LD4-binding Proteins

To identify LD4-binding proteins, GST–LD4 fusion protein was incubated with a smooth muscle tissue lysate. The bound proteins were subjected to SDS-PAGE, transferred to PVDF membrane, and visualized with Ponceau S staining. Individual protein bands, absent in a GST control precipitation, were excised and subjected to microsequencing. Two peptide sequences from the 150-kD protein were identical to clathrin. One peptide sequence from each of the 97- and 80-kD bands matched with two forms of the recently identified guanine nucleotide exchange factor (GEF), PAK-interacting exchange factor (PIX; Manser et al., 1998), also called COOL (Bagrodia et al., 1998), or p85SPR (Woo et al., 1997). The selective binding of PIX and clathrin to paxillin LD4 was confirmed by Western blotting (Fig. 3 A). Multiple immunoreactive bands in the PIX immunoblot are consistent with the existence of multiple isoforms (Manser et al., 1998). Importantly, deletion (dl) of LD4 from a GST–paxillin fusion protein spanning the NH$_2$ terminus (GST–54-313 dl LD4) resulted in a complete loss of PIX binding (Fig. 3 B). It should be noted that this fusion protein retains the ability to bind FAK via LD2 (see Fig. 5 B). Four peptides derived from the p95 protein (p95PKL) were sequenced. Peptides one through three were 100% identical to the previously reported KIAA0148 gene (Nagase et al., 1995). However, the cDNA for KIAA0148 en-codes a 50-kD protein. This cDNA was used to probe a human leukemia library to isolate a full-length p95PKL clone. The full-length p95PKL cDNA contained coding sequence for the fourth peptide derived from the original microsequencing analysis. Analysis of other cDNA clones isolated in the screen indicates that there are multiple splice variants of p95PKL. These will be described elsewhere.

Sequence Analysis of p95PKL

The PKL gene encodes a 757-aa protein with a calculated molecular weight of $\sim$85 kD. It is closely related to CAT (Bagrodia et al., 1999), the 53-kD protein KIAA0148 (Na-
gase et al., 1995), and G protein-coupled receptor kinase-interacting protein (GIT-1), a β-adrenergic receptor kinase-binding protein (Premont et al., 1998). An alignment of PKL, CAT, and KIAA0148 is presented in Fig. 4. Although p95PKL, CAT, and KIAA0148 show significant identity within the first 471-aa, p95CAT contains a 50-aa deletion (corresponding to aa 415–464 of PKL and KIAA0148; see Fig. 4 B). PKL is more distantly related to PKL, CAT, and KIAA0148 is presented in Fig. 4. Aligned in the regulation of secretory pathways and mitosis 34% identity, 53% conservation), that have been implicated in the regulation of secretory pathways and mitosis (Turner et al., 1998). Located within aa residues 11–44 of this domain is a GCS family zinc-finger characterized by a C_{6}C_{3}H_{2} zinc-coordinating residue scheme. This zinc-finger is contained within a consensus SAT motif (suppressor of Arf t mutation) spanning aa residues 11–60 (Zhang et al., 1998). The fact that the p95PKL related proteins GIT-1 and ASAP have been confirmed to exhibit ARF–GAP activity (Brown et al., 1998c; Premont et al., 1998) predicts PKL, CAT, and KIAA0148 will likely share this function.

The p95 family contains three ankyrin repeats spanning aa 167–199, 200–232, and 134–166. The combined p95PKL, ARF–GAP, and ankyrin repeat region shows 26% identity to and 43% conservation with the tumor suppressor BRCA1-associated RING domain protein, BARD1 (Ayi et al., 1998). A possible role for Ca^{2+} calmodulin regulation of p95PKL is suggested by the presence of an EF hand (aa 417–430), and two partial IQ motifs on the COOH terminus (aa 654–671 and 710–718). The EF hand is present in p95PKL, KIAA0148, and GIT-1, but is absent from the PKL splice variant CAT (Fig. 4) and ASAP. The COOH terminus of p95PKL and p95CAT shares homology to a region of myosin maintained on clip and hook proteins such as restin/CLIP-170, giantin, EEA1, and hook-1, which are involved in vesicle and organelle linkage to microtubule networks (data not shown).

Table I. Alignment of Internal Peptide Sequences from Paxillin LD4-binding Proteins with Previously Described Proteins

| Protein | Peptide sequence |
|---------|-----------------|
| p97     | SLVDTVYALKDEVGELR 17 |
| PIX     | SLVDTVYALKDEVGELR 605 |
| p80     | KPSDEEFAVR 10 |
| p95     | LLSLGAQANFFHPFE 14 |
| KIAA0148 | LLSLGAQANFFHPE 165 |
| p95     | VPFLYPYNEYSATR 14 |
| KIAA0148 | VPFLYPYNEYSSTR 327 |
| p95     | TPVDR 7 |
| KIAA0148 | TPVDR 208 |
| p95     | LQTLSNENTLNR 12 |
| KIAA0148 | LLGDKDN* |
| p150    | ERESYVELIFALA 15 |
| Clathrin | ERESYVELIFALA 1178 |
| p150    | EEEGETQ11VYGQ 15 |
| Clathrin | EEEGETQ11VYGQ 1636 |

GST–LD4 binding proteins from a chicken gizzard smooth muscle cell lysate were separated by SDS-PAGE, transferred to PVDF membrane, and visualized by Ponceau S staining. Multiple peptides from proteins of 150, 97, 95, and 80 kD were obtained by microsequencing (see Materials and Methods). Note that peptides from p97 and p80 matched with PIX family members. Underlined sequence designates deviation from published sequences.

*Denotes the terminal aa in the KIAA0148 sequence.

PAXIN LD4 Precipitates a Protein Complex Containing PAX, PAK, and Neck

PIX was previously identified through a high affinity association with PAK complexed with the p21 GTPase, Cdc42. Both PAK and Cdc42 are involved in reorganization of the actin cytoskeleton in response to soluble growth factors, and both PAK and PIX have been colocalized with Paxillin at focal complex sites formed in response to activation of Cdc42 and Rac (Manser et al., 1998). Therefore, we sought to determine if PAK was associated with the LD4 motif of Paxillin. Lysates from rat brain were incubated with rat brain lysate and binding proteins were precipitated using glutathione agarose beads. The coprecipitating proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with an antibody raised against PIX (Materials and Methods). PIX binding was restricted to the GST–LD4 fusion protein. Note that multiple isoforms of PIX have been reported, with brain expressing the most variants. The adaptor protein Crk failed to bind to any of the GST fusion proteins. A similar experiment was performed with gizzard smooth muscle lysate and probed with anticalchin antibody. Clathrin bound exclusively to the paxillin GST–LD4 motif. (B) To confirm a role for LD4 in PIX-binding, the experiment was repeated using a fusion protein of the NH_{2} terminus of paxillin containing a deletion of LD4 (54–313 dl LD4). PIX failed to bind to the LD4 deletion mutant. Crk served as a negative control.

Figure 3. PIX and clathrin bind selectively to paxillin LD4. (A) Individual GST–LD domains of paxillin were incubated with rat brain lysate and binding proteins were precipitated using glutathione agarose beads. The coprecipitating proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with an antibody raised against PIX (Materials and Methods). PIX binding was restricted to the GST–LD4 fusion protein. Note that multiple isoforms of PIX have been reported, with brain expressing the most variants. The adaptor protein Crk failed to bind to any of the GST fusion proteins. A similar experiment was performed with gizzard smooth muscle lysate and probed with anticalchin antibody. Clathrin bound exclusively to the paxillin GST–LD4 motif. (B) To confirm a role for LD4 in PIX-binding, the experiment was repeated using a fusion protein of the NH_{2} terminus of paxillin containing a deletion of LD4 (54–313 dl LD4). PIX failed to bind to the LD4 deletion mutant. Crk served as a negative control.
cubated with the individual GST–paxillin LD motifs and binding of PAK to the precipitates was evaluated by Western blotting. PAK was precipitated by GST–LD4 and the GST–54-313 paxillin fusion containing LD2–5 (Fig. 5, A and B). PAK was not detected in individual LD1, 2, 3, or 5 precipitates. The filter was reprobed with an antibody to the focal adhesion protein p130Cas. No binding of Cas to any of the paxillin fusion proteins was detected (Fig. 5 A).

The SH3-SH3-SH3-SH2 adaptor protein Nck interacts with PAK via the second SH3 domain (Bokoch et al., 1996; Galisteo et al., 1996). The ability of paxillin to coprecipitate Nck with PAK was confirmed by blotting the GST–54-313 paxillin precipitate with anti-Nck antibody (Fig. 5 B). Importantly, deletion of LD4 from this GST–paxillin fusion protein (GST–54-313 dl LD4) resulted in complete loss of PAK and Nck binding (Fig. 5 B), whereas the binding of FAK to this fusion protein was diminished but not eliminated, due to the presence of LD2, a second FAK binding site (Fig. 1). No binding of the adaptor protein Crk was detected in any of the precipitates.

A specific association of PIX and PAK with paxillin in vivo was confirmed by coimmunoprecipitation from brain lysate (Fig. 6). The failure of the largest PIX isoform to precipitate efficiently with paxillin suggests that intact paxillin may discriminate between PIX family members for binding. No binding of p130Cas to either the paxillin or control IgG precipitate was detected.

The Binding of PIX and PAK to Paxillin Is Mediated by p95PKL

To determine whether p95PKL, PIX, or PAK interact directly with paxillin, metabolically labeled proteins were synthesized using a cell-free coupled transcription/translation system. 35S-labeled p95PKL, PIX, and PAK were incubated with GST fusion proteins of PIX, PAK, paxillin NH2 terminus (aa 54–313), or GST. Bound proteins were detected by SDS-PAGE followed by fluorography. Previously reported interactions between PAK and PIX were confirmed using this cell-free system (Fig. 7 B). However, neither of these proteins were precipitated by the paxillin NH2 terminus (Fig. 7 B). In contrast, p95PKL was enriched in both the GST paxillin NH3 terminus and GST–PIX precipitates, but was not precipitated by either GST or GST–PAK (Fig. 7 B). These data support a role for p95PKL in mediating the association of paxillin with PIX. p95PKL also bound to GST full-length paxillin but not to a GST fusion protein of the four LIM domains of paxillin (data not shown). The association of p95PKL with paxillin...
and PIX provides a potential mechanism for recruiting PAK to the NH2 terminus of paxillin.

To confirm that the paxillin LD4 motif supports p95PKL binding, a precipitation assay was performed using GST–LD4 and the NH2 terminus of paxillin GST–54–313 were incubated with rat brain lysate and precipitated on glutathione agarose beads. Coproprecipitating proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with antibodies to PAK and p130Cas. PAK was precipitated by GST fusions comprising paxillin 54–313 and LD4. No binding of p130Cas to any of the paxillin fusion proteins was detected. (B) A specific role for the LD4 motif in binding PAK and Nck was confirmed by comparison of binding to paxillin 54–313 and the paxillin 54–313 dl LD4 mutant in precipitation assays. Deletion of LD4 eliminated the binding of PAK and Nck. FAK binding was reduced, but not completely eliminated, consistent with the ability of FAkt to bind to paxillin LD2 (Fig. 1), which remains intact in the 54–313 dl LD4 mutant.

Figure 5. PAK and the SH3-SH3-SH2-adaptor protein Nck bind to paxillin LD4. (A) Individual GST–LD motif and paxillin GST–54–313 were incubated with rat brain lysate and precipitated on glutathione agarose beads. Coproprecipitating proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with antibodies to PAK and p130Cas. PAK was precipitated by GST fusions comprising paxillin 54–313 and LD4. No binding of p130Cas to any of the paxillin fusion proteins was detected. (B) A specific role for the LD4 motif in binding PAK and Nck was confirmed by comparison of binding to paxillin 54–313 and the paxillin 54–313 dl LD4 mutant in precipitation assays. Deletion of LD4 eliminated the binding of PAK and Nck. FAK binding was reduced, but not completely eliminated, consistent with the ability of FAkt to bind to paxillin LD2 (Fig. 1), which remains intact in the 54–313 dl LD4 mutant.

Kinase Activity Associated with the LD4 Motif Precipitate Is Stimulated by Activated Cdc42

PIX has been suggested to mediate PAK activation by Cdc42 (Manser et al., 1998). To determine if the PAK associated with LD4 was enzymatically active, precipitation kinase assays using rat brain lysate were performed in the presence of the exogenous substrate for PAK, myelin basic protein. Phosphorylation of myelin basic protein was detected in coprecipitates of GST–LD4 in the absence of exogenously added GTPase, but not with GST–LD1 precipitates (Fig. 8 A). The associated kinase activity was stimulated further by the addition of GTP-loaded GST–Cdc42. Enhanced phosphorylation of coprecipitating proteins of 100–120, and 70 kD was also noted (Fig. 8 A). Although Rac stimulates PAK in vivo, GST–Rac, as well as GST–Rho, were unable to activate LD4 associated PAK in this in vitro assay, consistent with previous reports (Bagrodia et al., 1995).

Previously, we have demonstrated that the GST paxillin NH2 terminus precipitates both tyrosine and serine kinase activity from tissue and cell lysates (Bellis et al., 1997). The tyrosine kinase has been identified as FAK, while the serine kinase is currently unidentified. These kinases phosphorylate the paxillin fusion protein on residues that are also phosphorylated on paxillin in vivo in response to integrin-mediated cell adhesion (Bellis et al., 1995, 1997; Schaller and Parsons, 1995), as well as a coprecipitating 95-kD protein that was heavily phosphorylated on both tyrosine and serine residues (Bellis et al., 1997). To investigate the possibility that the p95 phosphoprotein was PKL and/or PIX, the precipitation kinase assay was repeated with GST–54–313 and B GST–54–313 dl LD4 (Fig. 8 B). Deletion of LD4 completely eliminated the 95- and 68-kD phosphorylated bands, consistent with the loss of binding of the p95PKL/PIX/PAK complex. In contrast, the 120-kD band that previously has been confirmed to be FAK was reduced, but not eliminated, consistent with the binding data presented in Fig. 5 B.

To determine if phosphorylation of the coprecipitating proteins caused dissociation of any of the proteins, the complex was washed extensively after the kinase reaction, before electrophoresis. The profile of precipitating phosphoproteins was identical to the unwashed sample (Fig. 8 B), indicating the paxillin/p95PKL/PIX/PAK complex is not perturbed by phosphorylation in this assay.

The Paxillin-related Protein, Hic-5, Binds the p95PKL/PIX/PAK Complex

Hic-5, a paxillin superfamily member (Shibumma et al., 1994; Brown et al., 1998b), may act as a functional antagonist of paxillin in vivo (Fujita et al., 1998; Thomas et al., 1999). The highest level of homology between the two pro-
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Proteins occurs within the LD motifs and the LIM domains (Brown et al., 1996). Hic-5, like paxillin, is targeted to focal adhesions via the LIM domains, and also binds vinculin, FAK/FRNK (FAK-related nonkinase), and Pyk2 (Fujita et al., 1998; Hagmann et al., 1998; Matasuya et al., 1998; Thomas et al., 1999). To test whether Hic-5 also precipitates a p95PKL/PIX/PAK complex, the NH2 terminus of Hic-5 (aa 1–226; Thomas et al., 1999) was expressed as a GST fusion protein and used in precipitation experiments. Western blot analysis of proteins precipitated by the Hic-5 NH2 terminus confirmed the binding of FAK, PAK, Nck, and PIX (Fig. 9). p130Cas served as a negative control. Direct binding of the Hic-5 NH2 terminus to p95PKL was detected by using 35S-labeled, in vitro translated p95PKL protein (Fig. 9).

**p95PKL Subcellular Localization**

To evaluate the subcellular distribution of p95PKL, the protein was expressed as a GFP fusion protein in CHO.K1 fibroblast cells. GFP–PKL localized to small focal adhesions associated with the ends of actin stress fibers (Fig. 10, **Figure 7**). p95PKL directly binds to paxillin LD4 and PIX. To identify the components of the LD4-binding complex that bound directly to paxillin, p95PKL, p85PIX, and PAK were 35S metabolically labeled in vitro by coupled transcription/translation and tested for their ability to bind GST fusion proteins of paxillin (aa 54–313), PIX, and PAK. (A) Translation products of p95PKL, PIX, and PAK. (B) PAK bound to PIX as previously reported. However, no binding of either of these proteins to paxillin 54–313 or GST was detected. In contrast, p95PKL bound to both the paxillin and PIX fusion proteins. No binding of p95PKL to PAK or GST was detected. (C) Deletion of LD4 from the paxillin NH2 terminus fusion protein eliminated the binding to p95PKL. GST–LD4 was sufficient for p95PKL binding. These data provide evidence that p95PKL functions to link paxillin and PIX to PAK. (D) Two regions of p95PKL spanning aa 1–576 and 448–757, respectively, were generated by coupled in vitro transcription/translation and tested for paxillin and PIX binding. PIX bound selectively to the NH2 terminus of p95PKL, containing PBS1 and the ARF–GAP and ankyrin domains, while paxillin bound to the COOH-terminal region containing PBS2. (E) To demonstrate an association in vivo between paxillin and p95PKL, GFP–p95PKL was transfected into COS 7 cells. After lysis, a paxillin precipitate and a mouse IgG control precipitate were subjected to SDS-PAGE, followed by Western blotting with antibodies to GFP, paxillin, and p130Cas. GFP–p95PKL was coprecipitated with paxillin, but not with the control Ig. No binding of p130Cas to either the paxillin or control IgG precipitate was observed. IgHC, immunoglobulin heavy chain; Ly, cell lysate.

**Figure 7.** p95PKL directly binds to paxillin LD4 and PIX. To identify the components of the LD4-binding complex that bound directly to paxillin, p95PKL, p85PIX, and PAK were 35S metabolically labeled in vitro by coupled transcription/translation and tested for their ability to bind GST fusion proteins of paxillin (aa 54–313), PIX, and PAK. (A) Translation products of p95PKL, PIX, and PAK. (B) PAK bound to PIX as previously reported. However, no binding of either of these proteins to paxillin 54–313 or GST was detected. In contrast, p95PKL bound to both the paxillin and PIX fusion proteins. No binding of p95PKL to PAK or GST was detected. (C) Deletion of LD4 from the paxillin NH2 terminus fusion protein eliminated the binding to p95PKL. GST–LD4 was sufficient for p95PKL binding. These data provide evidence that p95PKL functions to link paxillin and PIX to PAK. (D) Two regions of p95PKL spanning aa 1–576 and 448–757, respectively, were generated by coupled in vitro transcription/translation and tested for paxillin and PIX binding. PIX bound selectively to the NH2 terminus of p95PKL, containing PBS1 and the ARF–GAP and ankyrin domains, while paxillin bound to the COOH-terminal region containing PBS2. (E) To demonstrate an association in vivo between paxillin and p95PKL, GFP–p95PKL was transfected into COS 7 cells. After lysis, a paxillin precipitate and a mouse IgG control precipitate were subjected to SDS-PAGE, followed by Western blotting with antibodies to GFP, paxillin, and p130Cas. GFP–p95PKL was coprecipitated with paxillin, but not with the control Ig. No binding of p130Cas to either the paxillin or control IgG precipitate was observed. IgHC, immunoglobulin heavy chain; Ly, cell lysate.

**Figure 8.** Paxillin fusion proteins precipitate kinase activity that is stimulated by activated Cdc42. (A) GST–LD1 and LD4 fusion proteins were incubated with brain lysate and the washed precipitate subjected to in vitro kinase assays in the presence of GTPγS or GDP-loaded p21 GTPases. GST–LD4 precipitated kinase activity in the absence of added p21 as judged by phosphorylation of exogenous myelin basic protein and phosphorylation of coprecipitating proteins migrating between 70 and 120 kD. The kinase activity associated with LD4 was further stimulated by the addition of GTP-loaded Cdc42. (B) GST–54-313 previously has been shown to precipitate both tyrosine kinase (FAK) and serine kinase activity, resulting in phosphorylation of the paxillin fusion protein and proteins of 70, 95, and 120 kD. Repeating the assay with GST–54-313 dl LD4 failed to generate phosphorylated proteins of 70 and 95 kD consistent with the absence of p95PKL and PAK from these precipitates. Reduction in the intensity of the 120-kD band is consistent with the partial loss of FAK binding to the LD4 deletion mutant. Washing of the precipitates following the in vitro kinase reaction failed to release any of the coprecipitating phosphoproteins.
A and B, arrowheads), and to paxillin-containing focal adhesions (Fig. 10, C and D). GFP–paxillin also localized to focal adhesions at the ends of actin stress fibers (Fig. 10, E and F). Some labeling of both GFP–PKL and GFP–paxillin along the stress fibers was also noted. GFP alone demonstrated a diffuse cytoplasmic and nuclear staining with no enrichment at the ends of actin stress fibers (Fig. 10, G and H).

**A Potential Role for Paxillin LD4 in Cytoskeletal Organization and Migration**

To examine a role for the LD4 motif of paxillin in p21 GTPase and PAK-dependent cytoskeletal reorganization such as lamellipodia formation (Dharmawardhane et al., 1997), SH-SY5Y neuroblastoma cells were transfected with either wild-type chicken paxillin or an LD4 deletion paxillin mutant. Cells expressing wild-type paxillin were generally more spread and contained a much more organized actin stress fiber network than cells expressing the LD4 deletion mutant, although both constructs localized to focal adhesions as determined by immunofluorescence staining with a chicken paxillin-specific antiserum (Fig. 11, A and B, arrowheads). Actin was visualized with rhodamine phalloidin (Fig. 11, C–H). Serum-starved cells (Fig. 11 E, wild-type paxillin transfectants; and F, LD4 deletion transfectants) were stimulated with insulin-like growth factor-1 (IGF-1) to induce lamellipodia (Leventhal et al., 1997). Cells expressing wild-type chicken paxillin exhibited numerous, large lamellipodia in response to 5 min exposure to IGF-1 (Fig. 11 G, arrowheads). In contrast, the cells expressing the LD4 mutant formed fewer, substantially smaller lamellipodia (Fig. 11 H, arrowheads).

FAK and PAK have a role in stimulating cell migration (Cary et al., 1996, 1998; Adam et al., 1998; Klemke et al., 1998). To test whether the interaction of FAK and PAK with paxillin through the LD4 motif is potentially important for FAK- and/or PAK-mediated cell motility, GST or GST–LD4 fusion protein was microinjected into NIH3T3 cells at the edge of a wounded monolayer of cells cultured on glass coverslips. A comparison of the rate of migration of injected cells into the wound indicated a statistically significant reduction in the rate of migration of cells injected with GST–LD4, as compared with control GST-injected cells (Fig. 12). Taken together, these data support a role for the paxillin LD4 motif in coordinating actin cytoskeleton dynamics at the plasma membrane.
We previously identified a novel leucine-rich repeating sequence within the NH₂ terminus of paxillin, termed paxillin LD motifs (Brown et al., 1996). These motifs are implicated in the binding of paxillin to vinculin, FAK, and the E6 oncoprotein from papillomavirus (Brown et al., 1996; Tong et al., 1997; Vande Pol et al., 1998). In this study, we have demonstrated that individual LD motifs can function independently to support the differential binding of the focal adhesion proteins vinculin and FAK. Several proteins were identified that were precipitated by paxillin LD4. These include clathrin and a new 95-kD, ARF–GAP domain- and ankyrin repeat-containing protein. PAK, Nck, and members of the Cdc42/Rac1 guanine nucleotide-exchange factor family PIX/COOL (Bagrodia et al., 1998; Manser et al., 1998) also associated with LD4. In vitro binding experiments using isolated proteins demonstrated that p95PKL binds directly to paxillin and PIX. Since direct binding of PAK to paxillin was not observed, the precipitation of PAK by paxillin LD4 from cell and tissue lysates likely occurs as a result of its association with PIX (Fig. 7; Bagrodia et al., 1998; Manser et al., 1998). Similarly, it is expected that LD4 precipitation of Nck is a result of its stable association with PAK (Bokoch et al., 1996; Galisteo et al., 1996). The coprecipitation of endogenous PIX family members and PAK with paxillin from brain lysate (Fig. 6), as well as previous reports detailing coprecipitation of PIX and PAK (Bagrodia et al., 1998; Manser et al., 1998), provides evidence for the existence of these interactions in vivo.

P95PKL is a member of a family of ARF–GAP proteins that include the closely related GIT-1, CAT, and KIAA0148, as well as the more distantly related ASAP1. Both GIT-1 and ASAP1 exhibit ARF–GAP activity and have been implicated in membrane remodeling events associated with Arf function. GIT-1 binds the β-adrenergic receptor kinase and is involved in coordinating receptor endocytosis (Premont et al., 1998), while ASAP, the largest family member, which also binds Src and Crk, has been localized to the plasma membrane where it is suggested to coordinate membrane trafficking associated with cytoskeletal remodeling or cell growth (Brown et al., 1998c). KIAA0148 is a smaller (50 kD) protein, containing highly conserved ARF–GAP and ankyrin-repeat regions, but lacking the more diverse COOH terminus domain, which
also bound to the NH2 terminus of Hic-5, suggesting con-
al., 1999). The p95PKL/PIX/PAK/Nck protein complex
FRNK, vinculin, and CSK (Fujita et al., 1998; Thomas et
ditional features with paxillin, including focal adhesion
al., 1998b). One family member, Hic-5, shares several ad-
LD motifs and COOH-terminal LIM domains (Brown et

Paxillin itself is a member of a larger family that is char-
acterized primarily by the conservation of NH2-terminal
motifs and COOH-terminal LIM domains (Brown et al., 1998b). One family member, Hic-5, shares several addi-
tional features with paxillin, including focal adhesion
targeting via the LIM domains and binding to FAK, FRNK, vinculin, and CSK (Fujita et al., 1998; Thomas et al.,
1999). The p95PKL/PIX/Pak/Nck protein complex also bound to the NH2 terminus of Hic-5, suggesting con-
servation of function of the LD motifs of these two pro-
teins. In contrast, unlike paxillin, Hic-5 is not tyrosine
phosphorylated during cell adhesion and consequently is
unable to bind the SH2-domain-containing adaptor, Crk
(Thomas et al., 1999). This suggests that Hic-5 and paxillin
may act as functional antagonists with regards to Crk-depen-
dent signaling events, such as cell migration (Klemke et al., 1998) and activation of the Ras–MAP kinase pathway
via C3G. It will be important to determine the relative
contribution of each of these multifamily members to par-
ticular cellular events and signal transduction pathways.

What is the functional significance of a paxillin, p95PKL
ARF–GAP protein association? A role for ARF1 and ARF6 proteins in cytoskeletal reorganization, including
cortical actin reorganization during cell spreading is ap-
parent (Van Aelst and D’Souza-Schorey, 1997; Frank et al., 1998; Sasaki and Takai, 1998; Song et al., 1998). The
capacity of paxillin to assemble a PAK-containing com-
plex through the ARF–GAP proteins PKL and CAT pro-
vides a possibility for the integration of ARF and Rho
family signal transduction at the cytoskeleton. In this re-
gard, we and others recently have determined that paxillin
cycles in an ARF-dependent manner, between the trans-
Golgi/endo-somal network and focal adhesions (Norman et al., 1998; Brown, M.C., and C.E. Turner, unpublished ob-
servations). One possible model would predict that regula-
tion of different ARF family members by ARF–GAPs
such as p95PKL determines the subcellular localization of
paxillin and other focal complex/adhesion components,
including PAK, PIX Cdc42, and vinculin (Erickson et al.,
1996; Norman et al., 1998; our unpublished observations).
Thus, cell adhesion or growth factor stimulation would
promote the release of paxillin and associated proteins from a perinuclear vesicle compartment, permitting tar-
geting to sites of cell matrix interaction, and thereby con-
tributing to the Cdc42/Rac/Rho paradigm. Interestingly,
clathrin was also precipitated specifically by paxillin LD4.
Although clathrin has not been observed in focal adhe-
sions, a-actinin and the vitronectin receptor have been re-
ported to bind clathrin (Merisko et al., 1988; De Deyne et
al., 1998). It is unknown at this time whether the binding of
clathrin to paxillin is direct or indirect. However, clathrin
does contain a well conserved PBS (Brown et al., 1998b).
The functional consequence of clathrin binding to paxillin
remains to be determined, but it also may be associated
with a role for p95PKL ARF–GAP activity and PAK in vesicular transport (Dharmawardhane et al., 1997), en-
docytosis (Brown et al., 1998c), and membrane trafficking
to the leading edge during cell motility or in response to
growth factors (Breyscher and Aguado-Velasco, 1998a,b).
It is also a potential factor in the redistribution of paxillin
to the trans-Golgi/endo-somal network in epithelial cells,
where colocalization of paxillin and clathrin has been ob-
served (Brown, M.C., and C.E. Turner, unpublished ob-
servations).

Stimulation of p21 GTPase activity in response to integ-
рин-mediated cell adhesion (Clark et al., 1998) or growth
factors results in reorganization of the actin cytoskeleton
and the formation of distinct sites of cell attachment with
the extracellular matrix. Rac and Cdc42 activation leads to
the formation of small focal complexes at the base of
lamellipodia and filopodia, respectively, while larger focal
adhesciones associated with robust actin stress fibers are
the hallmark of Rho activity (Ridley and Hall, 1992; Ridley et
al., 1992). Focal complexes and focal adhesions contain
several common components including transmembrane in-
tegrin receptors and intracellular proteins, such as paxillin,
vinculin, and FAK (Nobes and Hall, 1995). PAX has been
suggested as the protein that recruits PAK to focal com-
pleses (Manser et al., 1998), but the protein responsible
for PIX targeting has not been identified. It has been pro-
posed that paxillin serves an adaptor function at these sites
of adhesion, using the LIM domains for focal adhesion tar-
geting (Brown et al., 1996; Thomas et al., 1999) and the
NH2 terminus in a scaffolding capacity, recruiting multiple
signaling components such as the tyrosine kinases FAK,
PYK2, Src, and Csk (Sabe et al., 1994; Turner and Miller,
1994; Schaller and Parsons, 1995; Tachibana et al., 1995;
Salgia et al., 1995). In view of the binding of a p95PKL/
PIX/PAK complex to the NH2 terminus of paxillin, and the
localization of a pool of PKL to focal complexes/adhes-
ces in CHO.K1 cells, future experiments will be directed
towards evaluating a potential role for p95PKL in recruit-
ing PAK and PIX to the paxillin-rich sites of cell adhesion.
In support of a role for paxillin in P21 GTPase- and
PAK-dependent cytoskeletal reorganization, we found
that overexpression in neuroblastoma SH-SYSY cells of a
paxillin mutant lacking the LD4 motif caused an overall
reduction in actin cytoskeletal organization in comparison
to wild-type paxillin-expressing cells, and substantially re-
duced lamellipodia formation in these cells in response to
IGF-1. Overexpression of PAK in neuronal PC12 cells
also stimulates lamellipodia in the absence of IGF-1
(Obermeier et al., 1998). This response, which requires
structural elements within both the NH2- and COOH-ter-
minal region of PAK, but is not dependent on PAK kinase
activity, is inhibited by coexpression of dominant-negative
Rac or by interfering with the interaction between PAK
and PIX (Obermeier et al., 1998). Thus, the ability of
the paxillin LD4 deletion mutant to inhibit lamellipodia for-
mation in response to IGF-1 likely is due in part to the fail-
ure of a PAK/PIX complex to target effectively to the

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plasma membrane through an association with paxillin. Similarly, the well-spread phenotype of SH-SY5Y cells overexpressing wild-type paxillin, as compared with the LD4 deletion mutant, provides further evidence for a role for paxillin in Cdc42 and Rac-dependent regulation of cell spreading (Price et al., 1998). Finally, the absence of stress fibers in the paxillin LD4 deletion-expressing cells suggests that this mutant may also affect Rho-dependent signaling.

Regulated attachment and detachment of integrin receptors to the extracellular matrix, as well as reorganization of the actomyosin cytoskeleton, is required for cell movement (Burridge and Chrzanowska-Wodnicka, 1996). Recent reports have indicated a role for both PAK and FAK in cell migration (Ilic et al., 1995; Cary et al., 1996; Gilmore and Romer, 1996; Adam et al., 1998). A paxillin/FAK and/or a paxillin/PAK association at the plasma membrane may be required for the cytoskeletal changes necessary for efficient cell motility. Importantly, PAK has been shown to phosphorylate and inhibit MLCK (Sanders et al., 1999), thereby providing a potential mechanism by which PAK can promote a motile Cdc42/Rac phenotype while inhibiting the less motile morphology characterized by robust stress fibers resulting from Rho-dependent activation of MLCK (Burridge and Chrzanowska-Wodnicka, 1996). Thus, microinjection of the nontargeting GST–LD4 fusion protein into NIH3T3 cells may be exerting an inhibitory effect on cell migration by sequestering sufficient FAK, PAK, and Cdc42 or Rac1 in the cytoplasm, preventing efficient lamellipodia and filopodia formation and extension at the leading edge of the cells.

Cell spreading and cell migration have also been associated with increased kinase activity of FAK (Ilic et al., 1995; Cary et al., 1996). We previously have shown that the NH2 terminus of paxillin, when expressed as a GST fusion protein, precipitates FAK and serine kinase activities. The paxillin residues phosphorylated by these kinases in vitro are also major sites of paxillin phosphorylation during cell spreading (Bellis et al., 1997). Conversely, inhibition of paxillin tyrosine phosphorylation blocks cell spreading (Richardson et al., 1997). Since p95PKL is phosphorylated on both tyrosine and serine residues in vitro (Richardson et al., 1997), it will be important to determine whether p95PKL is a target for FAK and/or PAK during cell adhesion/migration. These data are also consistent with the observation that p95CAT, a PKL family member, is tyrosine phosphorylated during cell adhesion (Bagrodia et al., 1999). In conclusion, we have demonstrated that the LD motifs of paxillin exhibit differential binding to several molecules important in the regulation of actin cytoskeleton dynamics. This includes the identification and cloning of a novel protein, p95PKL, that links paxillin indirectly to PAK and shares homology with ARF–GAP proteins involved in vesicular transport. Future experiments will examine the role of paxillin in coordinating the potential cross-talk between these paxillin-binding proteins.

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