

# Identification of LIM3 as the Principal Determinant of Paxillin Focal Adhesion Localization and Characterization of a Novel Motif on Paxillin Directing Vinculin and Focal Adhesion Kinase Binding

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**Abstract.** Paxillin is a 68-kD focal adhesion phosphoprotein that interacts with several proteins including members of the src family of tyrosine kinases, the transforming protein v-crk, and the cytoskeletal proteins vinculin and the tyrosine kinase, focal adhesion kinase (FAK). This suggests a function for paxillin as a molecular adaptor, responsible for the recruitment of structural and signaling molecules to focal adhesions. The current study defines the vinculin- and FAK-interaction domains on paxillin and identifies the principal paxillin focal adhesion targeting motif. Using truncation and deletion mutagenesis, we have localized the vinculin-binding site on paxillin to a contiguous stretch of 21 amino acids spanning residues 143–164. In contrast, maximal binding of FAK to paxillin requires, in addition to the region of paxillin spanning amino acids 143–164, a carboxyl-terminal domain encompassing residues 265–313. These data demonstrate the presence of a single binding site for vinculin, and at least two binding sites for FAK that are separated by an intervening stretch of 100 amino acids. Vinculin- and FAK-binding activities within amino acids 143–164 were separable since mutation of amino acid 151 from a negatively charged glutamic acid to the uncharged polar residue glutamine (E151Q) reduced binding of vinculin to paxillin by >90%, with no reduction in the binding

capacity for FAK. The requirement for focal adhesion targeting of the vinculin- and FAK-binding regions within paxillin was determined by transfection into CHO.K1 fibroblasts. Significantly and surprisingly, paxillin constructs containing both deletion and point mutations that abrogate binding of FAK and/or vinculin were found to target effectively to focal adhesions. Additionally, expression of the amino-terminal 313 amino acids of paxillin containing intact vinculin- and FAK-binding domains failed to target to focal adhesions. This indicated other regions of paxillin were functioning as focal adhesion localization motifs. The carboxyl-terminal half of paxillin (amino acids 313–559) contains four contiguous double zinc finger LIM domains. Transfection analyses of sequential carboxyl-terminal truncations of the four individual LIM motifs and site-directed mutagenesis of LIM domains 1, 2, and 3, as well as deletion mutagenesis, revealed that the principal mechanism of targeting paxillin to focal adhesions is through LIM3. These data demonstrate that paxillin localizes to focal adhesions independent of interactions with vinculin and/or FAK, and represents the first definitive demonstration of LIM domains functioning as a primary determinant of protein subcellular localization to focal adhesions.

**C**ELLULAR adhesion to the extracellular matrix is critically involved in many processes including normal and transformed cell growth, migration, and metastasis, lymphocyte extravasation, and force transmission during muscle contraction (for review see Burridge, 1986; Hynes, 1992; Clark and Brugge, 1995). A com-

plex mechanism of cell association with the extracellular matrix has evolved and is governed predominantly by the integrin family of heterodimeric transmembrane glycoproteins (Hynes, 1992). These receptors mechanically couple to, and mediate signaling from, the extracellular matrix to the actin cytoskeleton through the direct association of intracellular molecules with the cytoplasmic domain of the integrin  $\beta$  subunit (Turner and Burridge, 1990; Luna and Hiitt, 1992). While  $\alpha$ -actinin and talin may be largely responsible for the physical coupling of integrins to the actin cytoskeleton, a number of other proteins contribute to the

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complex of cytoskeletal proteins concentrated at specialized sites of cell adhesion to the extracellular matrix known as focal adhesions (Burrige et al., 1988). Focal adhesion proteins customarily have been categorized as either structural molecules, such as the actin-binding proteins tensin and vinculin, or regulatory molecules, including zyxin, the focal adhesion tyrosine kinase (FAK)<sup>1</sup>, and paxillin (Turner and Burrige, 1990; Lo and Chen, 1994). The potential interplay of these various focal adhesion proteins has been studied in an effort to delineate the structure and to probe the function of the focal adhesion.

Vinculin is an abundant cytoskeletal protein present at cell-matrix and cell-cell contacts that interacts with  $\alpha$ -actinin, talin, F-actin, tensin, and paxillin (for review see Hemmings et al., 1995). An important role for vinculin in adhesion has been inferred by a number of studies, including the capacity of exogenous expression of vinculin to complement nonadherence of a vinculin-deficient embryonal carcinoma cell line (Samuels et al., 1993). Additionally, a role for vinculin in cell morphology, motility, and anchorage-dependent growth has been demonstrated using antisense suppression of vinculin expression in NIH3T3 cells (Fernández et al., 1993).

Paxillin was originally described as a 68-kD, vinculin-binding protein through the use of blot overlay assays using paxillin purified from smooth muscle (Turner et al., 1990). Further analyses using bacterially expressed glutathione-S-transferase (GST)-vinculin fusion proteins and purified paxillin have permitted the mapping of the paxillin-binding site on vinculin to a 22-amino acid region that is highly conserved in nematode, frog, chick, pig, and human vinculin (Wood et al., 1994). In the same study, transfection experiments indicated that the 22-amino acid paxillin-binding domain lies within a 50-amino acid region of the vinculin tail involved in the targeting of this protein to focal adhesions. As a result, it is generally accepted that paxillin localization to focal adhesions is, at least in part, due to an association with this abundant focal adhesion component.

Paxillin is phosphorylated on tyrosine residues to a high stoichiometry (20–30%) during various cellular events associated with cell adhesion, remodeling of the actin-based cytoskeleton, and growth control (for review see Turner, 1994). This increase in tyrosine phosphorylation of paxillin is accompanied by a similar elevation in the tyrosine phosphorylation of FAK, suggesting a direct correlation between FAK activation and paxillin phosphorylation (Burrige et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992; Turner et al., 1993, 1995).

Isolation of a cDNA encoding the paxillin protein has permitted the generation of GST-paxillin fusion proteins that have been used to identify binding sites for both vinculin and FAK within the amino-terminal half of the protein (Turner and Miller, 1994), as well as to determine the predominant site of tyrosine phosphorylation of paxillin by FAK in vitro (Bellis et al., 1995). Subsequent studies have firmly established that FAK associates directly with paxillin (Hildebrand et al., 1995; Tachibana et al., 1995).

1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; GST, glutathione-S-transferase; PBS, paxillin-binding subdomain; dl, deletion.

The paxillin cDNA revealed several additional motifs that have been implicated in protein-protein interactions further supporting the model that paxillin functions as an adaptor molecule directing focal adhesion dynamics. These modules include several potential tyrosine phosphorylation sites that are consensus SH2 domain-binding motifs. Tyrosine 118 is of particular interest, because phosphorylation of this residue by FAK forms a functional crk SH2-binding domain, consistent with data demonstrating an association between the v-crk SH2 domain and tyrosine phosphorylated paxillin in vivo and in vitro (Birge et al., 1993; Bellis et al., 1995; Schaller and Parsons, 1995). A short proline-rich region conforms very closely to the consensus sequence for an SH3-binding motif (Ren et al., 1993) and may be responsible for the interaction between paxillin and the SH3 domain of the tyrosine kinase c-src (Weng et al., 1993). Importantly, neither the tyrosine phosphorylated crk SH2-binding domain nor the proline-rich region are required for targeting paxillin to focal adhesions (Bellis et al., 1995). Finally, the carboxyl half of paxillin (amino acids 313–559) contains four LIM domains.

The LIM domains of paxillin are cysteine/histidine-rich motifs of 51 amino acids and are related to the larger family of zinc-finger proteins (for review see Sánchez-García and Rabbitts, 1994). LIM domains were first identified in the homeodomain-containing transcription factors *Lin-11*, *Isl-1*, and *Mec-3* (Sánchez-García and Rabbitts, 1994). These proteins are involved in cell lineage determination and pattern formation during embryogenesis. Subsequently, several groups of LIM-containing proteins have emerged. Members of the protooncogene rhombotin family of helix-loop-helix transcription regulators, associated with normal T cell development and T cell acute lymphocytic leukemia, have also been shown to contain LIM domains (Sánchez-García and Rabbitts, 1994). A novel role for LIM domains as protein dimerization motifs has been proposed (Rabbitts and Boehm, 1990). The existence of other classes of LIM-containing proteins that lack DNA-binding domains has emerged more recently. Paxillin is a member of this class that also includes the proteins zyxin and cysteine-rich protein, proteins that are associated with the cytoskeleton at focal adhesions (Sadler et al., 1992). The diversity of LIM protein families continues to grow with the characterization of a family of novel LIM-containing protein serine/threonine kinases (Okano et al., 1995).

The experiments presented in this manuscript offer a definition and molecular dissection of the vinculin- and FAK-binding sites on paxillin, and investigate the potential role for the multiple protein-interaction domains of paxillin in targeting paxillin to focal adhesions.

## Materials and Methods

### DNA Constructs

Chicken paxillin expression vectors were constructed by PCR amplification of selected regions of a paxillin cDNA template (Turner and Miller, 1994; Bellis et al., 1995) using primers incorporating 5'-BamHI and 3'-EcoRI restriction sites followed by introduction of the amplicon into the mammalian expression vector, pcDNA3 (Invitrogen, San Diego, CA), or the bacterial expression vector, pGEX2T (Pharmacia Fine Chemicals, Piscataway, NJ). Correct in-frame incorporation, or introduction of start or stop

codons, was verified by sequencing with vector-specific oligonucleotides. The paxillin cDNA, subcloned into M13, was used as a template for deletion and site-directed mutagenesis according to standard procedures (Kunkel, 1985).

### **GST Fusion Proteins**

To express the GST-paxillin fusion proteins, *Escherichia coli* (DH5 $\alpha$ ) transformed with the appropriate pGEX2T-paxillin construct were grown overnight, diluted 1:10, and grown for an additional 1.5 h. Protein expression was induced for 3 h by the addition of 0.1–0.5 mM isopropyl- $\beta$ -D-thiogalactopyranose. Cells were harvested by centrifugation at 10,000 g for 10 min. Fusion protein was purified by lysis of bacteria in Tris-buffered saline, pH 8.0, containing 2 mg/ml lysozyme, 0.1%  $\beta$ -mercaptoethanol, and protease inhibitors for 30 min at room temperature. Triton X-100 was added to 1%, and the cells were incubated on ice for 10 min. Bacterial cell wall debris was removed by centrifugation at 10,000 g for 15 min with the fusion protein recovered from the supernatant by incubation with glutathione-Sepharose 4B (Pharmacia Fine Chemicals) according to the manufacturer's instructions.

### **GST-Paxillin Binding Assays**

A lysate of embryonic day 18–20 chicken gizzard was prepared by homogenizing the tissue in 10 vol of lysis buffer containing 50 mM Tris/HCl, pH 7.6, 50 mM NaCl, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.1% mercaptoethanol, 1% Triton X-100, and a cocktail of protease inhibitors. The lysate was clarified at 100,000 g for 30 min. Aliquots of lysate (1 mg of protein) were incubated with the various GST-paxillin fusion proteins coupled to the glutathione-Sepharose 4B beads or with GST-glutathione-Sepharose 4B for 90 min, washed extensively in lysis buffer, and boiled directly in SDS-PAGE sample buffer. After electrophoresis, the proteins were transferred to nitrocellulose and probed with the appropriate antibody.

### **SDS-PAGE and Western Blotting**

SDS-PAGE electrophoresis was performed using standard procedures using either 10 or 12.5% polyacrylamide gels. Western immunoblotting and radioiodination of secondary antibodies were performed as described previously (Towbin et al., 1979; Turner et al., 1990). Antibodies used in this study were as follows: paxillin mAb 165 (Turner et al., 1990) and a chicken-specific polyclonal antiserum, Pax 1 (Turner and Miller, 1994); vinculin mAb Vin-11-5 (Sigma Chemical Co., St. Louis, MO); and FAK polyclonal antibody, BC3 (a generous gift from Dr. J. Thomas Parsons, University of Virginia, Charlottesville, VA).

### **Cell Culture and Transfection**

CHO.K1 cells (a generous gift from J. Couchman and A. Woods, University of Alabama at Birmingham) were cultured in modified Ham's F-12 (Mediatech, Washington, DC) supplemented with 10% (vol/vol) heat-inactivated, certified FBS (GIBCO BRL, Gaithersburg, MD) and 1% penicillin-streptomycin at 37°C in a humidified chamber with 5% CO<sub>2</sub>. Cells were grown on tissue-culture-treated plastic dishes and routinely passaged every 5 d by washing in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free PBS followed by gentle trypsin-EDTA treatment. CHO.K1 cells were transfected using LIPOFECTAMINE (GIBCO BRL) essentially as previously described (O'Toole et al., 1995) with minor modifications. Briefly, CHO.K1 cells were plated at a density of 3.5  $\times$  10<sup>5</sup> cells per 35-mm dish 24 h before transfection. 1  $\mu$ g of the respective paxillin-pcDNA3 construct and 5  $\mu$ g of LIPOFECTAMINE were diluted separately into 100  $\mu$ l of serum- and antibiotic-free Ham's F-12 medium followed by mixing of the DNA and lipid and incubation at room temperature for 15 min. During this incubation, the adherent CHO.K1 cells were washed twice with prewarmed, unsupplemented medium. Unsupplemented medium was then added to the DNA-lipid mixture to bring the volume to 1 ml, and the mixture was overlaid onto the cells for a 6-h incubation at 37°C. After 6 h, 1 ml of Ham's F-12 with 20% FBS and 1% penicillin-streptomycin was added, and the cells were incubated overnight. The cells were washed twice in complete medium, and then changed to complete Ham's F-12 with 1.5 mg/ml G418 (GIBCO BRL) after 24 h. After an initial phase of selection, the G418 concentration was reduced to 750  $\mu$ g/ml for maintenance of enriched populations of transfected cells. The cells were harvested, plated onto 13-mm glass coverslips, and processed for immunofluorescence analysis as has been described in detail elsewhere (Turner and Miller, 1994). The cells photographed are representative of each transfected cell population.

## **Results**

### **Mapping the Vinculin- and FAK-binding Sites on Paxillin by Truncation Mutagenesis**

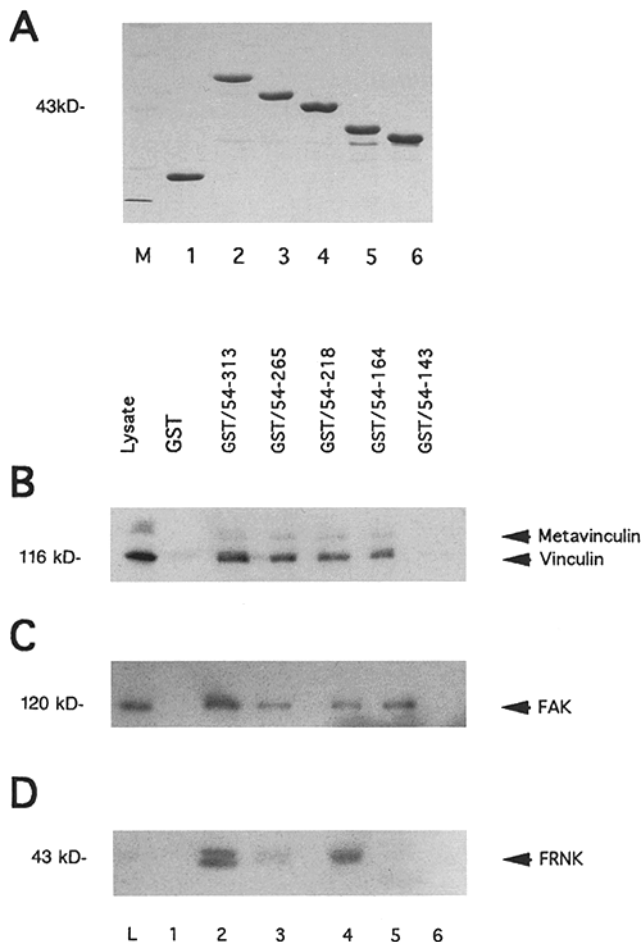
In a previous report, we identified a region of paxillin spanning amino acids 54–313 that, when expressed as a GST fusion protein, supports the binding of both vinculin and FAK (Turner and Miller, 1994). The inability of another abundant focal adhesion protein, talin, to bind to this fusion protein confirmed the specificity of these interactions. As an initial step to determine more precisely the binding sites for these two proteins, a series of carboxyl-terminal deletions of this region of paxillin was generated by PCR and expressed as GST fusion proteins as shown in Fig. 1 A. The ability of each of these fusion proteins to bind vinculin, metavinculin (a smooth muscle-specific isoform of vinculin) (Belkin et al., 1988), and FAK was tested in *in vitro* binding assays by incubating GST-paxillin fusion proteins with chicken gizzard smooth muscle lysate (Fig. 1, B–D) followed by Western blotting with antibodies to vinculin and FAK as described in Materials and Methods. The results of this analysis (Fig. 1) indicated that binding of vinculin, metavinculin, and FAK to paxillin was retained by the construct expressing paxillin residues 54–164 (lane 5), but that the shorter paxillin fusion protein spanning residues 54–143 lost the ability to bind all of these proteins (lane 6). Therefore, residues 143–164 of paxillin contain a binding site for these proteins. Binding of both phosphoisoforms of the noncatalytic variant of FAK (Schaller et al., 1993), known as FAK-related nonkinase (FRNK), generally mirrored that of FAK; however, variability in the binding of FRNK to residues 54–164 was noted, which may reflect a more complex association with paxillin or simply inefficient transfer of these proteins as a result of comigration on the gel of the GST-paxillin fusion protein.

As the interaction between vinculin and paxillin has been defined previously using blot overlay technology (Turner et al., 1990; Wood et al., 1994), the binding of purified, radioiodinated vinculin to the GST-paxillin fusion proteins immobilized on nitrocellulose was tested and yielded identical results (data not shown).

The amino-terminal limits of the vinculin- and FAK-binding sites were examined further. Consistent with the carboxyl-terminal truncations reported in Fig. 1, a GST-paxillin construct spanning residues 168–313 failed to precipitate vinculin or metavinculin (Fig. 2 A, lane 4), thus defining the binding site for vinculin on paxillin within amino acids 143–164. Surprisingly, the same construct (168–313) retained the capacity to bind FAK (Fig. 2 B, lane 5). Binding of FAK was only completely eliminated by combining this amino-terminal deletion with a carboxyl-terminal deletion from 266–313 (lane 6). These data suggest that there are potentially two discrete regions of paxillin separated by 97 amino acids that contribute to the FAK-binding site.

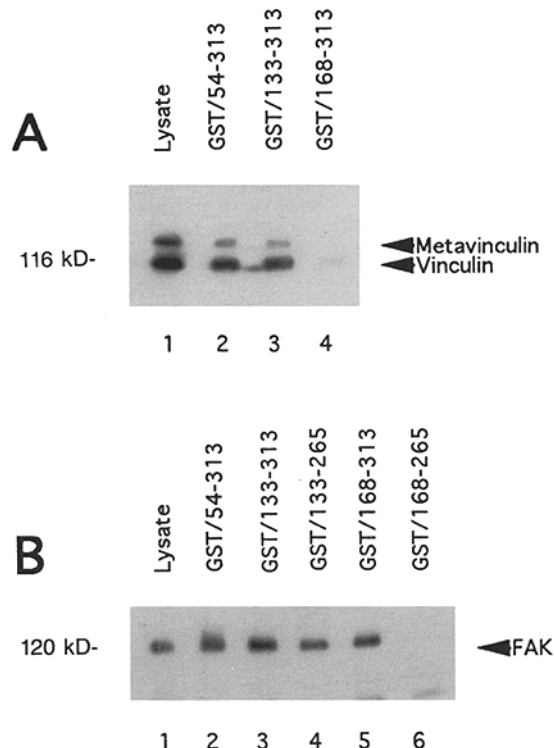
### **Molecular Dissection of the Vinculin- and FAK-binding Domains**

Further confirmation that amino acid residues 143–164 contained the vinculin-binding site was obtained by deleting the region of paxillin encompassing residues 134–167



**Figure 1.** Vinculin and FAK binding to carboxyl-terminal deletions of GST-paxillin (54–313). A series of carboxyl-terminal deletions of the vinculin- and FAK-binding region of paxillin (shown previously to be within residues 54–313 [Turner and Miller, 1994]) was generated by PCR and expressed as GST fusion proteins. **A** shows a Coomassie brilliant blue–stained gel of the purified GST fusion proteins used. These were coupled to glutathione–Sepharose 4B beads and incubated with smooth muscle tissue lysate. After extensive washing, the protein complexes were electrophoresed, and then blotted with antibodies to vinculin (note the vinculin antibody recognizes both vinculin [116 kD] and metavinculin [150 kD]) (**B**) or FAK/FRNK (**C** and **D**). The region of paxillin encoded by each fusion protein is listed at the top of the figure. Removal of residues 164–143 (compare lanes 5 and 6) resulted in the loss of binding of vinculin and FAK/FRNK.

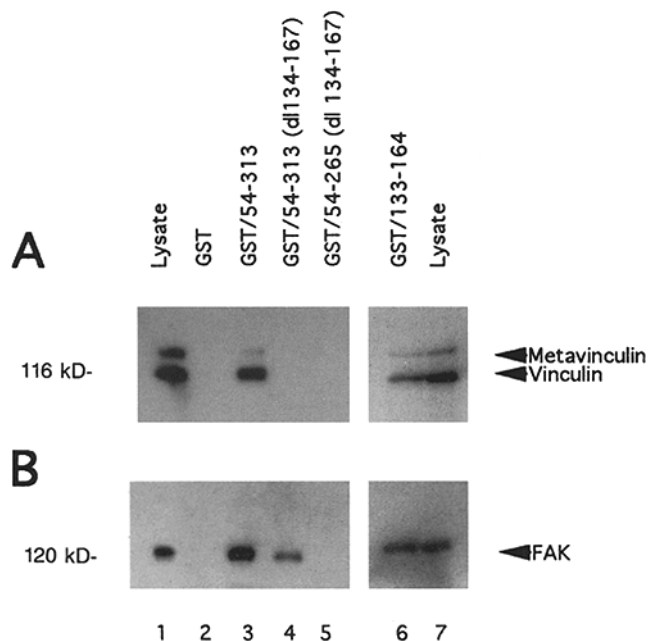
from the GST–54–313 construct by site-directed mutagenesis. As expected, this construct failed to coprecipitate vinculin or metavinculin (Fig. 3 *A*, lane 4). In contrast, the binding of FAK was retained, although at a reduced level when compared to 54–313 (Fig. 3 *B*, lanes 3 and 4). Consistent with the truncation mutagenesis binding data, further deletion of the carboxyl terminus was found to be necessary to completely abolish FAK binding (Fig. 3 *B*, lane 5), reinforcing the argument for the involvement of two regions of paxillin in the binding of FAK. To definitively demonstrate the capacity of amino acid residues 133–164 to support vinculin and FAK binding, this region was expressed as a GST fusion protein and incubated with



**Figure 2.** Two distant regions of the GST 54–313–paxillin fusion protein contain FAK-binding activity. Precipitation binding studies were performed using smooth muscle lysate as described in Materials and Methods, and precipitated proteins were probed with antibodies to vinculin (**A**) or FAK (**B**). (Lane 1) Total tissue lysate. (Lane 2) Paxillin fusion protein (54–313) containing binding sites for vinculin (and metavinculin) and FAK. While the amino-terminal deletion construct spanning residues 133–313 bound both vinculin and FAK (lane 3), an additional truncation to 168 (GST 168–313) failed to bind vinculin (**A**, lane 4) but retained significant FAK binding (**B**, lane 5). A combination of this amino-terminal truncation with a carboxyl-terminal truncation (GST 168–265) was necessary to eliminate FAK binding (**B**, lane 6). The carboxyl-terminal truncation alone (GST 133–265) was not sufficient to eliminate binding of FAK (**B**, lane 4).

chicken smooth muscle lysate; the resulting precipitate was blotted and probed by vinculin and FAK antibodies as above. This defined region of paxillin is capable of supporting the binding of vinculin and FAK (Fig. 3, *A* and *B*, lane 6).

To dissect further those residues important for vinculin binding from those necessary for FAK binding, we generated a limited number of point mutations within the region of paxillin spanning amino acid residues 143–164 and assayed them for their ability to bind metavinculin, vinculin, and FAK. A double point mutation of the negatively charged aspartate residue 146 to an uncharged polar asparagine (D146N) and positively charged arginine residue 147 to an uncharged polar glutamine (R147Q), as well as the single point mutation of the negatively charged glutamate residue 151 to an uncharged polar glutamine (E151Q), reduced the vinculin binding to <10% of control levels (Fig. 4 *A*, lanes 2 and 3). The double mutation (D146N/R147Q) had a similar, if not greater, effect on reducing the binding of FAK (Fig. 4 *B*, lane 2), suggesting

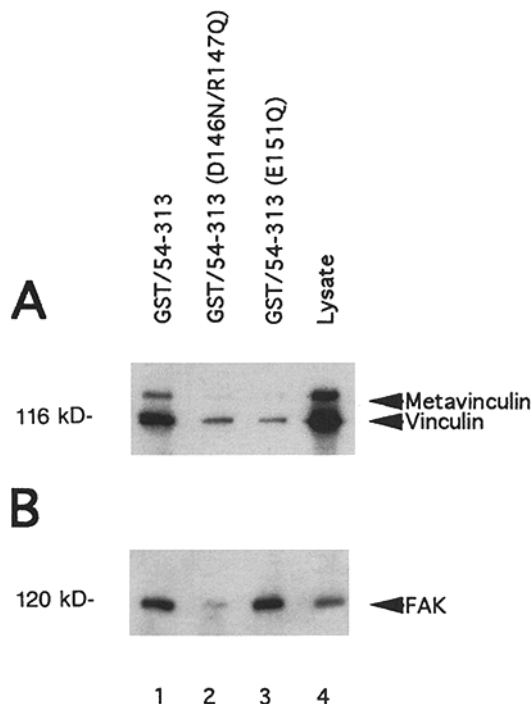


**Figure 3.** Internal deletion of the vinculin binding site. The vinculin binding site (residues 134–167 of paxillin) as defined by carboxyl- and amino-terminal deletions described in Figs. 1 and 2 was deleted from GST 54–313 by site-directed mutagenesis. This construct lost the capacity to bind vinculin (A, lane 4) but retained FAK binding (B, lane 4). Consistent with a model involving two regions of paxillin in FAK binding, a carboxyl-terminal deletion of residues 266–313 combined with the internal deletion was necessary to eliminate FAK-binding capacity (B, lane 5). Importantly, the internal binding site (133–164) is capable of supporting the binding of both vinculin and FAK (lane 6) in and of itself. *dl*, deletion.

that these residues contribute significantly to the binding of both vinculin and FAK. In contrast, the E151Q mutation did not cause any reduction in FAK binding when compared with the control fusion protein (Fig. 4 B, lane 3). Therefore, while E151Q is critical for normal vinculin binding to paxillin, it is not an essential determinant of FAK binding, whereas paxillin residues D146 and R147 contribute to both vinculin and FAK binding. These binding data are summarized in Table I.

#### Vinculin, FAK, and Paxillin Interaction Domain Sequence Analysis

As the regions on paxillin that interact with vinculin (134–167) and FAK (134–167 and 265–313) were relatively defined, we sought to determine whether these regions of paxillin were homologous to other known proteins. A BLAST search (Altschul et al., 1990) of SWISS-PROT Release 32.0 revealed a strikingly high homology with one protein, the TGF $\beta$ 1- and hydrogen peroxide-inducible LIM protein, HIC-5 (Shibanuma et al., 1994). Interestingly, three particular regions of the paxillin and HIC-5 molecules are highly conserved. These domains include a region of paxillin within the vinculin/FAK-binding domain (134–167), which is 64% identical and 72% conserved to HIC-5 amino acids 63–89; the more carboxyl-terminal FAK-binding domain (265–313), which is 60% identical



**Figure 4.** Point mutations can discriminate between the vinculin- and proximal FAK-binding sites. The charge on three amino acids (146, 147, and 151) was neutralized by site-directed mutagenesis within the vinculin/FAK-binding sites. These mutants were used in precipitation-binding studies as described. The double point mutation D146N/R147Q reduced the binding of vinculin by >90% (A, lane 2) when compared with the control fusion protein (A, lane 1). The single point mutation E151Q resulted in a similar reduction in vinculin binding (A, lane 3). This mutation failed to perturb the binding of FAK (B, lane 3). In contrast, the double mutation significantly reduced FAK binding (B, lane 2).

and 67% conserved to HIC-5 amino acids 139–198; and the four LIM domains with 62% identity and 78% conservation to HIC-5 amino acids 211–444 (data not shown). We speculate on the basis of such a defined conservation of motifs that HIC-5, a protein potentially involved in cellular senescence (Shibanuma et al., 1994), is a paxillin family member. When the conserved sequences of paxillin and HIC-5 are examined more closely, three segments of the vinculin- and FAK-binding regions mapped on paxillin have a remarkably conserved character within HIC-5. These three segments that are 85% identical between paxillin and HIC-5 and are likely sequence elements mediating the interaction between paxillin, vinculin, and FAK (Table II) contain a 13-amino acid repeated motif. Paxillin also contains within its extreme amino terminus an additional repeat of this motif. A function for this repeat is not known. We have named these 13 amino acid repeats paxillin LD motifs on the basis of the conserved character of the tandem leucine and aspartate amino acid residues in both paxillin and HIC-5. Preliminary studies investigating a functional role for the LD motifs of HIC-5 indicate that HIC-5 is capable of interacting with vinculin and FAK (Turner, C.E., M.C. Brown, and S.M. Thomas, unpublished observations).

Table I. GST/Paxillin-binding Activity for Vinculin and FAK

GST/Paxillin fusion protein	Binding protein	
	Vinculin	FAK
Paxillin amino acids encoded by cDNA		
54-313	++++	++++
54-265	++	+
54-218	++	+
54-164	++	+
54-143	-	-
113-313	++++	+++
168-313	-	+++
133-265	ND	++
168-265	-	-
54-313 (dl 134-167)	-	++
54-296 (dl 134-167)	ND	+++*
54-265 (dl 134-167)	-	-
133-164	+++	++++
54-313 (D146N/R147Q)	-†	-†
54-313 (E151Q)	-†	++++
GST	-	-

\*Data not shown.

†Binding reduced by >90%.

### Contribution of the Vinculin- and FAK-binding Domains to Paxillin Focal Adhesion Targeting

The definition of the vinculin- and FAK-binding sites on paxillin using in vitro-binding analyses allowed for the determination of the potential role for vinculin and FAK in localizing paxillin to focal adhesions. To examine the effect of deletion of the vinculin-binding domain on paxillin targeting, CHO.K1 fibroblasts were transfected with full-length wild-type chicken paxillin cDNA or the full-length construct encoding a deletion of amino acids 134-167, followed by immunofluorescence analysis of paxillin localization using a chicken-specific polyclonal antibody, named Pax 1, raised against purified avian smooth muscle paxillin (Turner et al., 1990). The full-length chicken paxillin molecule localized efficiently to focal adhesions (Fig. 5 A). Double staining with rhodamine-phalloidin to decorate actin filaments confirmed the localization of paxillin to the ends of stress fibers (Fig. 5 B). To verify colocalization of wild-type chicken paxillin with vinculin, CHO.K1 cells transfected with wild-type paxillin were fixed and immunostained using the paxillin polyclonal antibody (Fig. 5 C) and a vinculin mAb (Fig. 5 D). A field containing a non-transfected cell (*asterisk*), which is vinculin positive, was photographed to document the species specificity of the paxillin antiserum. Transfection and immunoanalysis of the paxillin construct containing the 134-167 deletion re-

Table II.

### Conserved LD Motifs of Paxillin and HIC-5

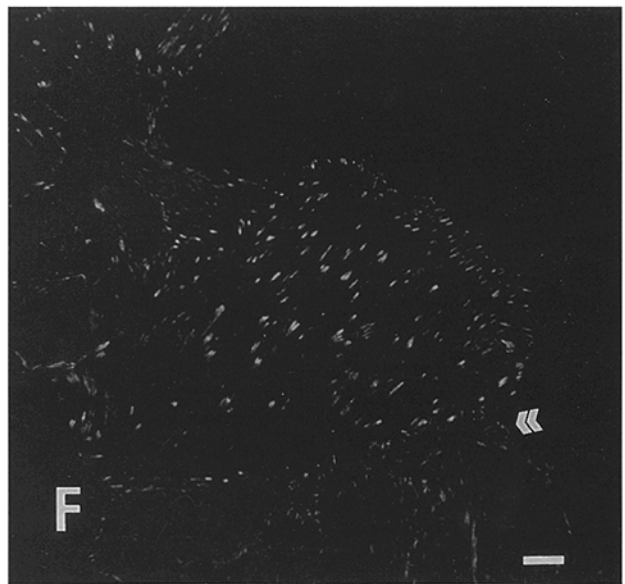
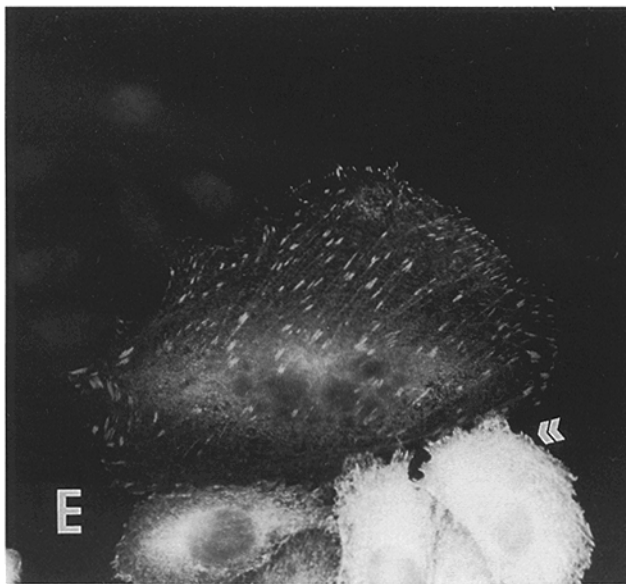
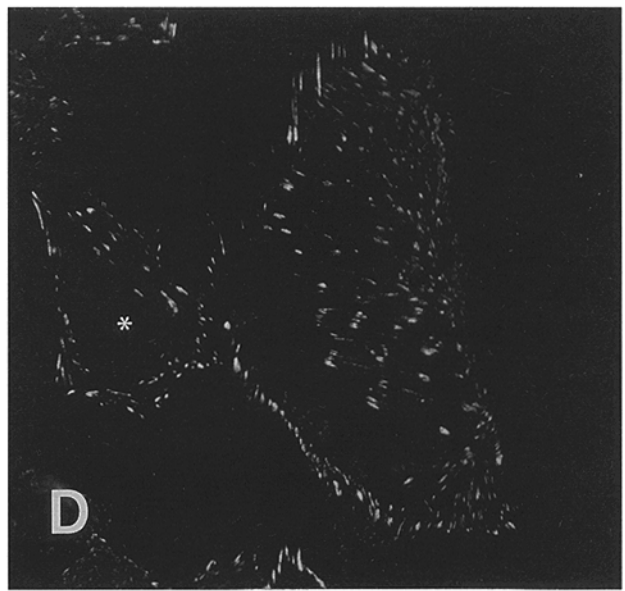
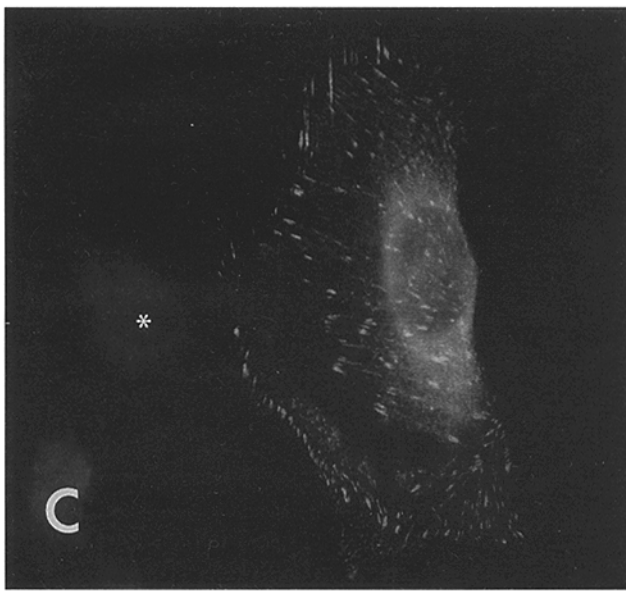
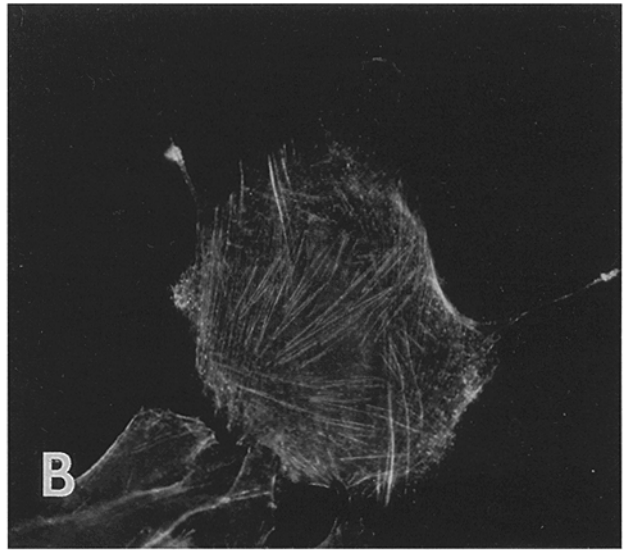
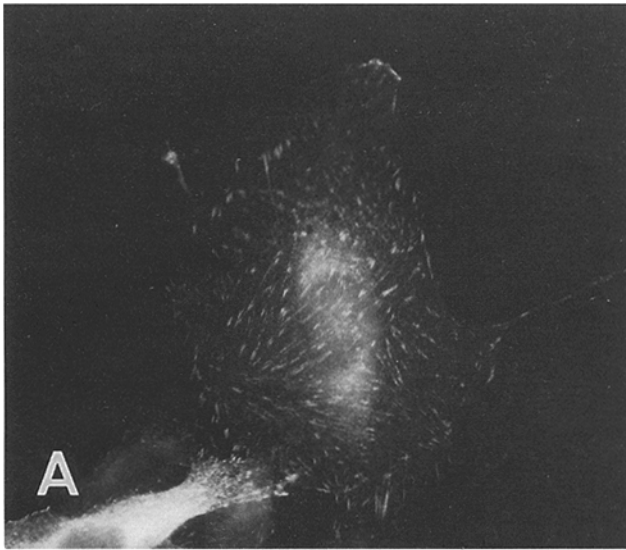
PAXILLIN	D	<b>L</b>	<b>D</b>	A	L	L	A	D	L	E	S	T	T	LD1
PAXILLIN	E	<b>L</b>	<b>D</b>	R	L	L	L	E	L	N	A	V	Q	LD2
HIC-5	E	<b>L</b>	<b>D</b>	R	L	L	Q	E	L	N	A	T	Q	
PAXILLIN	E	<b>L</b>	<b>D</b>	E	L	M	A	S	L	S	D	F	K	LD3
HIC-5	E	<b>L</b>	<b>D</b>	R	L	M	A	S	L	S	D	F	R	
PAXILLIN	Q	<b>L</b>	<b>D</b>	T	M	L	G	S	L	Q	S	D	L	LD4
HIC-5	S	<b>L</b>	<b>D</b>	T	M	L	G	L	L	Q	S	D	L	

Alignment of the paxillin primary amino acid sequence with the potential homologue HIC-5. The primary amino acid sequence of each of the four 13-amino acid repeats are identical between avian and human paxillin, except for a conservative threonine to serine substitution at position 4 within LD4 (data not shown). These repeats have been named LD motifs on the basis of the conservation of the leucine (L) and aspartate (D) doublet (*boxed and in bold*) in each of the repeats of paxillin and HIC-5. Additionally, these motifs share a combination of leucine and/or methionine residues at positions 5 and 6, as well as a carboxyl-terminal leucine at position 9 (*boxed*).

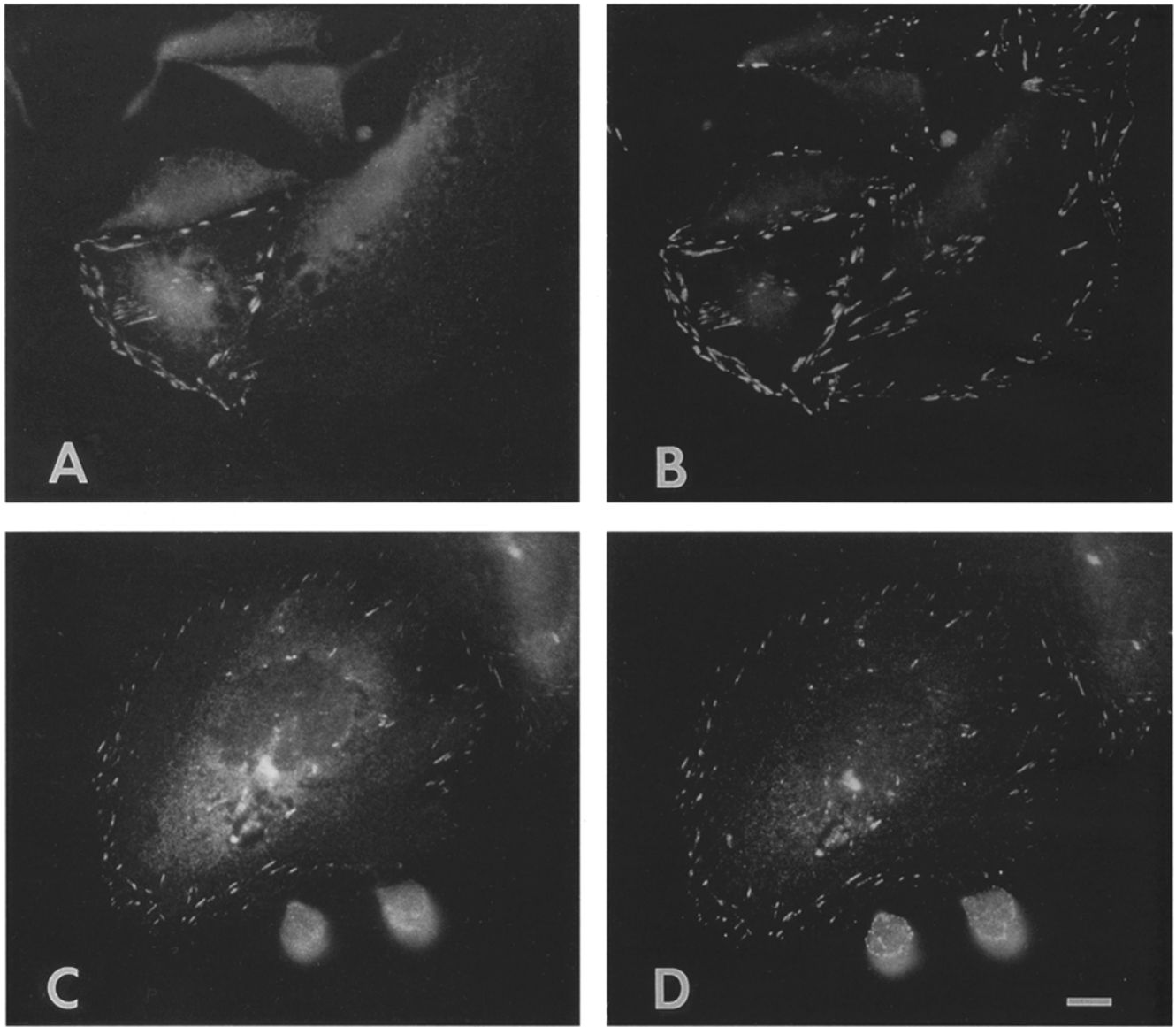
vealed strong focal adhesion localization (Fig. 5 E), demonstrating that the targeting of paxillin to focal adhesions is independent of vinculin binding. Interestingly, overexpression of full-length paxillin with the 134-167 deletion did not block focal adhesion formation (Fig. 5 E, *double arrow*) or vinculin targeting (Fig. 5 F, *double arrow*), although the focal adhesions did appear to be smaller than wild-type contacts.

Paxillin containing the point mutations that affected vinculin and FAK binding in vitro were next examined to confirm the nonessential nature of vinculin binding for paxillin focal adhesion localization (Fig. 6). Indeed, paxillin E151Q localized to sites of focal adhesion (Fig. 6 A) and did not affect vinculin targeting (Fig. 6 B). Paxillin D146N/R147Q, which had a severely reduced capacity to bind FAK, as well as vinculin, in in vitro-binding assays (Fig. 4), was examined by immunofluorescence analysis to determine if this impaired ability to interact with each of these focal adhesion proteins diminished paxillin targeting in vivo. Despite the effective absence of these binding sites, mutagenesis of the vinculin- and FAK-binding domain did not eliminate paxillin or FAK localization (Fig. 6, C and D).

**Figure 5.** Focal adhesion targeting of a paxillin construct that lacks the vinculin-binding domain. CHO.K1 fibroblasts were transfected with chicken paxillin constructs, followed by indirect immunofluorescence analysis using a chicken-specific paxillin polyclonal antiserum (Pax 1), and double labeled with either rhodamine-phalloidin to decorate actin stress fibers or a vinculin mAb (Vin 11-5). Cells transfected with full-length wild-type paxillin were stained with anti-paxillin antiserum (A and C) and double labeled with rhodamine-phalloidin (B) or vinculin antibody (D). The asterisk denotes a nontransfected cell. Cells were transfected with full-length paxillin containing the 134-167 vinculin-binding site deletion, stained with anti-paxillin antiserum (E), and double labeled with vinculin antibody (F). This construct localizes efficiently to focal adhesions. The double arrows point to the presence of paxillin and vinculin-containing focal adhesions even in cells overexpressing the paxillin construct. Bar, 5  $\mu$ m.







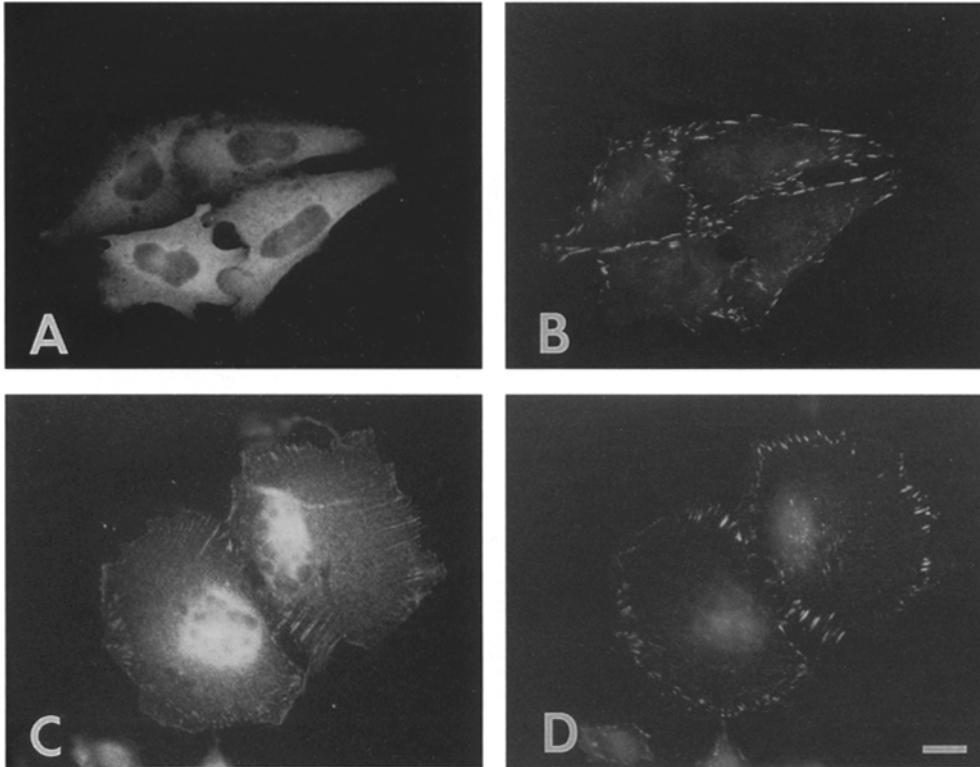
**Figure 6.** Effect of point mutations within the common vinculin- and FAK-binding region of paxillin on focal adhesion targeting. CHO.K1 fibroblasts were transfected with chicken paxillin constructs followed by indirect immunofluorescence analysis. Cells transfected with full-length E151Q paxillin were stained with anti-paxillin antiserum (*A*) and double labeled with anti-vinculin antibody (*B*). Cells transfected with full-length D146N/R147Q paxillin were stained with anti-paxillin antiserum (*C*) and double labeled with 2A7, an FAK mAb (*D*). Bar, 5  $\mu\text{m}$ .

To exclude completely the potential involvement of the amino terminus in targeting paxillin to focal adhesions, we transfected CHO.K1 fibroblasts with a pcDNA3 construct encoding amino acids 1–313 of paxillin. This region of the protein contains the SH3- and crk SH2-binding domains, as well as the entire vinculin- and FAK-binding domains, but excludes the four carboxyl-terminal LIM domains. When the transfectants were examined by immunofluorescence using the paxillin polyclonal antiserum, cytoplasmic staining but no focal adhesion targeting was detectable (Fig. 7 *A*), whereas double labeling of these transfectants with a vinculin mAb confirmed the presence of focal adhesions in these cells (Fig. 7 *B*). The failure of this carboxyl-terminal truncated paxillin molecule to target to focal adhesions confirms that, in the absence of the LIM domains, the vin-

culin- and FAK-binding domains cannot function to direct paxillin localization. Conversely, a region of paxillin spanning amino acid residues 168–559 that lacks the combined vinculin- and FAK-binding domain but contains the four LIM domains was capable of targeting to focal adhesions (Fig. 7 *C*). Interestingly, this truncated paxillin molecule localizes to both focal adhesions and extends partially along actin stress fibers, while vinculin in the same cell is restricted to focal adhesions (Fig. 7 *D*).

These data demonstrate that the predominant means of targeting paxillin to focal adhesions lies outside of the amino-terminal vinculin- and FAK-binding domains and, consistent with past analyses (Bellis et al., 1995), outside of the potential SH3-binding domain and crk SH2-binding domain. Consequently, the LIM domains of paxillin are





**Figure 7.** Inability of paxillin lacking the carboxyl-terminal LIM domains to localize to focal adhesions. CHO.K1 fibroblasts were transfected with a chicken paxillin construct spanning residues 1–313, a region containing the vinculin- and FAK-binding region but lacking the LIM domains, followed by indirect immunofluorescence analysis. A cytosolic distribution was observed with Pax 1 staining (A), while double labeling with a vinculin mAb confirmed that these cells had focal adhesions (B). Examination of cells transfected with a construct encoding residues 168–559, a region containing the LIM domains, revealed efficient targeting to focal adhesions as well as some stress fiber staining (C, Pax 1; D, Vin 11-5). The distribution of 168–559 in heterogeneous populations of transfected cells was somewhat variable in that, in addition to focal adhesion staining, some cells exhibited greater staining along stress fibers than others. Bar, 5  $\mu$ m.

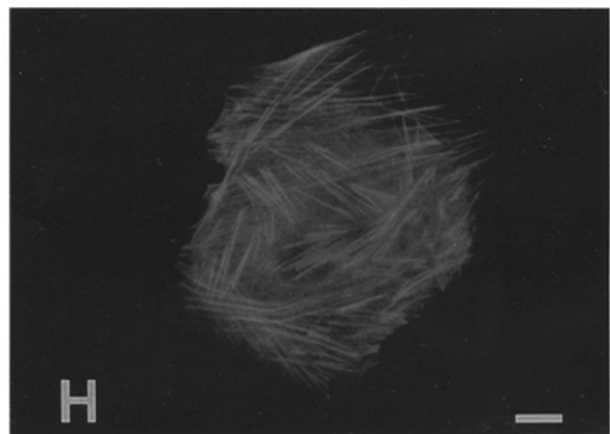
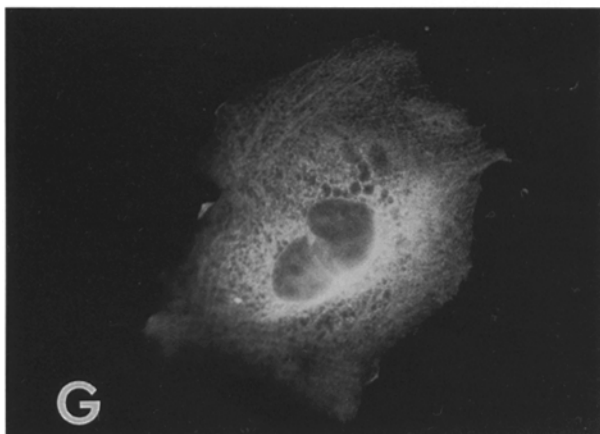
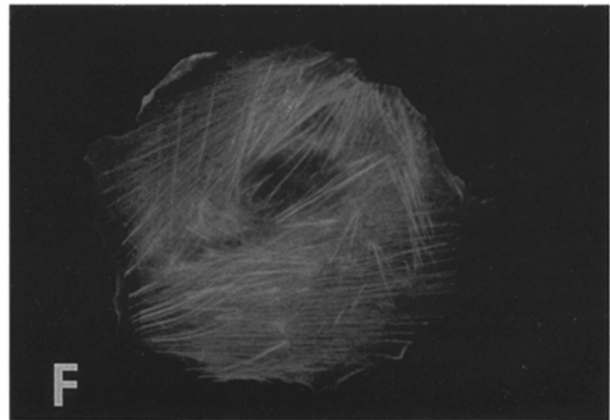
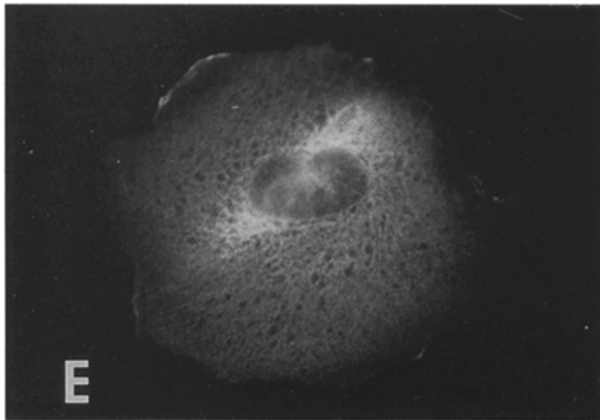
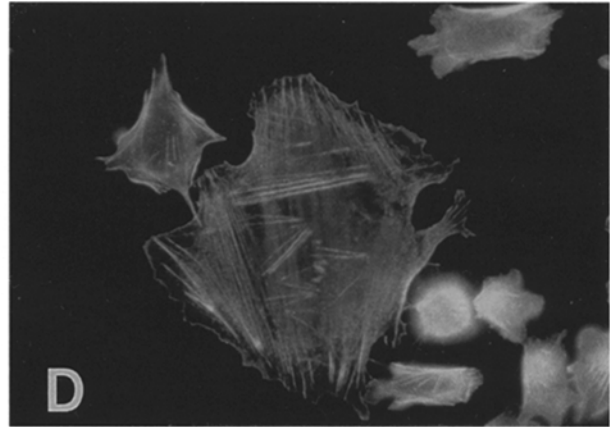
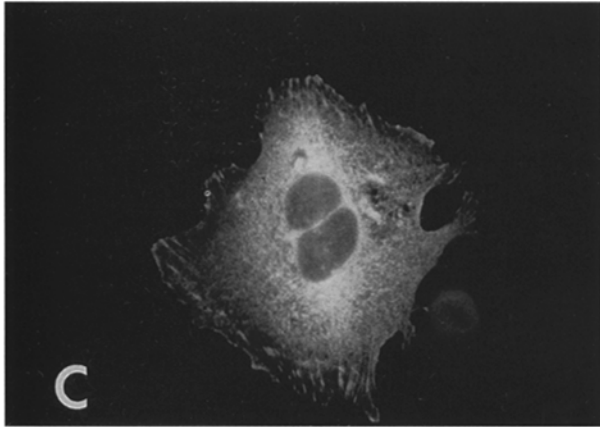
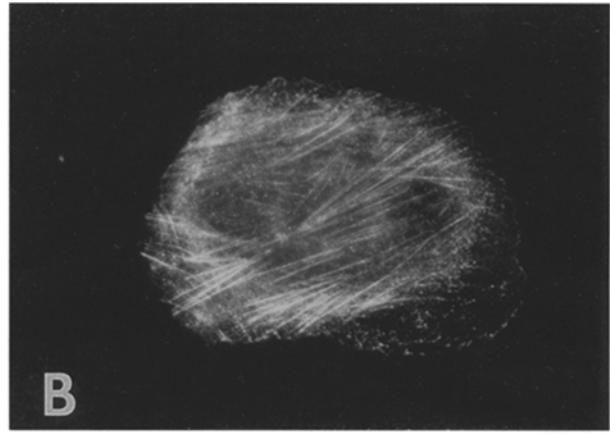
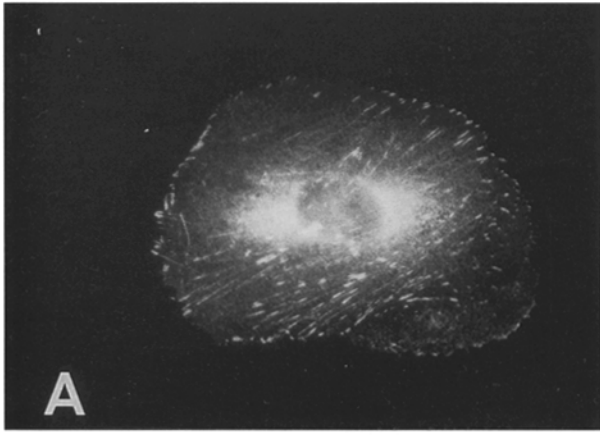
implicated as the primary mechanism of paxillin focal adhesion targeting.

### ***A Role for the LIM Domains of Paxillin as a Focal Adhesion–targeting Motif***

To characterize the involvement of the LIM domains of paxillin in focal adhesion targeting, sequential truncation of the four carboxyl-terminal LIM domains was produced in the pcDNA3 construct, 54–559. This protein lacks the potential SH3-binding motif but targets efficiently to focal adhesions (Bellis et al., 1995; Fig. 8, A and B). Expression and distribution of paxillin mutants encoded within these constructs was determined by immunofluorescence analysis using the paxillin polyclonal antiserum and rhodamine-phalloidin. A paxillin molecule with a deletion of LIM4 localized efficiently to focal adhesions (Fig. 8, C and D). However, further truncation by the removal of LIM3 resulted in the elimination of targeting (Fig. 8, E and F). This implicates LIM3 as the primary mechanism of paxillin focal adhesion localization. Importantly, the molecule encoded in this construct retains the vinculin- and FAK-binding domains, yet it is unable to support localization of paxillin to focal adhesions.

LIM domains are complex structures composed of two zinc fingers. Each zinc finger is formed and stabilized through the chelation of one molecule of zinc by a combination of four cysteine, histidine, or aspartate amino acid

residues. LIM motifs conform to the following consensus:  $C^1X_2C^2X_{16-23}H^3X_2C^4X_2C^5X_2C^6X_{16-21}C^7X_2(C,H,D)^8$ . The folding of this domain can be perturbed readily through the introduction of point mutations of individual zinc coordinating residues (Feuerstein et al., 1994). Consequently, paxillin constructs were generated that contained point mutations of zinc-chelating residues within both the first and second zinc fingers of LIM1, LIM2, or LIM3, in the context of the full-length molecule, thus disrupting the structure of an individual LIM domain. Immunofluorescence analysis of expression of the full-length paxillin LIM1 double mutant (H<sup>4</sup>349I/C<sup>5</sup>352A) in CHO.K1 fibroblasts revealed efficient localization to focal adhesions that was comparable to wild type (Fig. 9, A and B). Expression of full-length paxillin LIM2 double mutant (H<sup>3</sup>405I/C<sup>5</sup>411A) in CHO.K1 fibroblasts revealed localization to focal adhesions (Fig. 9, C and D). However, the targeting was frequently less efficient when compared with other targeting competent mutants, as assessed by the relative intensity of the focal adhesion staining. This mutant paxillin molecule displays a proportionate decrease in colocalization with vinculin as compared with colocalization of the 134–167 paxillin deletion mutant with vinculin (Fig. 5). Finally, we generated a similar mutation of LIM3 (C<sup>4</sup>467A/C<sup>5</sup>470A) in the full-length paxillin molecule. This modification eliminated targeting to focal adhesions (Fig. 9, E and F), consistent with the LIM3 truncation mutant and confirming a role for the third LIM domain of paxillin in targeting this focal adhe-



sion protein. That paxillin has four LIM domain double zinc finger motifs suggests the possibility of a complex interplay between the LIM domains in protein function. The decrease in efficiency of localization of the LIM2 zinc finger double mutant suggests a potential cooperativity between LIM2 and LIM3 in targeting paxillin to focal adhesions. The role of the LIM domains in paxillin focal adhesion targeting is potentially complex, and further studies will be required to determine the precise interplay amongst the LIM domains in facilitating subcellular localization. Nonetheless, when a paxillin construct encoding a full-length molecule with LIM3 deleted is expressed in CHO.K1 cells, a complete elimination of targeting is observed (Fig. 9, *G* and *H*), further confirming LIM3 as the principal determinant of paxillin subcellular localization to focal adhesions. The results of the expression analysis are presented in Table III.

## Discussion

Generally, it has been accepted that paxillin is targeted to focal adhesions in part through an interaction with vinculin and/or FAK (Wood et al., 1994; Hildebrand et al., 1995). Through the process of truncation, deletion, and site-directed mutagenesis, we have defined the vinculin- and FAK-binding sites on paxillin in an effort to examine the contribution of these interactions to paxillin focal adhesion localization. A single region localized to amino acids 143–167 supported vinculin binding; whereas this region, in addition to paxillin residues 265–313, contributed to the binding of FAK. Examination of these binding sites revealed the presence of four novel, repeated motifs of 13 amino acids within the primary amino acid sequence of paxillin that we have named LD motifs (Table II). The first LD repeat is located on the extreme amino terminus of paxillin, the second resides within the combined vinculin- and FAK-binding region (143–168), and the third and fourth within the second FAK-binding region (265–313). A role for the fourth LD in supporting FAK binding has been excluded by producing an additional carboxyl-terminal truncation of the 54–313 (deletion [dl] 134–167) to 54–296 (dl 134–167), which was capable of binding FAK (unpublished observation). Since these repeats are found in a variety of proteins, including dystrophin, myosin heavy chain, and  $G_{\alpha}$  guanine nucleotide regulatory protein subunits, we suspect that these are novel protein–protein interaction motifs of general importance.

Previously, the paxillin-binding site on vinculin was mapped to a 22-amino acid region spanning residues 978–1,000 within the vinculin tail (Wood et al., 1994), which was sufficient for paxillin binding but not for focal adhesion localization of vinculin. An additional carboxyl-terminal 28 amino acids were required for efficient localization of the tail domain of vinculin to focal adhesions in NIH3T3

cells. This raised the possibility that paxillin is targeted to focal adhesions in part through an association with vinculin. Interestingly, transfection of a full-length paxillin molecule containing a deletion of the vinculin-binding site (amino acids 134–167) into CHO.K1 fibroblasts revealed no effect on paxillin focal adhesion localization, demonstrating the ability of paxillin to target in the absence of vinculin binding (Fig. 5). The capacity of paxillin to target to focal adhesions independent of an interaction with this major structural component was at first surprising. However, during the course of this study, reports using F9 and embryonic stem cell vinculin-null cells revealed, in fact, enhanced labeling of focal adhesions for paxillin (Volberg et al., 1995), although these cells were less well spread than wild-type counterparts and were 2.4-fold more motile (Coll et al., 1995), confirming that paxillin targeting to focal adhesions can occur independently of an interaction with vinculin. Additionally, the inability of paxillin to be recruited to focal adhesions through an association with vinculin may provide an explanation for the failure of paxillin to colocalize with vinculin at sites of cell–cell adhesion (Turner et al., 1990).

The carboxyl terminus of FAK supports both paxillin binding and focal adhesion localization, with the paxillin-binding site and the focal adhesion targeting sequence reported to be distinct (Hildebrand et al., 1995). This suggested that FAK localization to focal adhesions is independent of binding to paxillin and that FAK is likely involved in the recruitment of paxillin to focal adhesions. These conclusions are in conflict with a recent study examining the interaction between FAK and paxillin and subsequent focal adhesion localization of FAK (Tachibana et al., 1995). A detailed molecular dissection of the paxillin-binding site on FAK revealed the involvement of two regions of FAK with high homology to the region of vinculin shown previously to interact with paxillin (Tachibana et al., 1995). The vinculin sequence is contiguous, whereas the FAK sequences are separated by 91 amino acids. Consequently, this region of homology was divided into two independent domains that were named paxillin-binding subdomain (PBS) 1 and 2. Interestingly, a recently identified FAK family member, PYK2 (Lev et al., 1995; Sasaki et al., 1995; Avraham et al., 1995; Li et al., 1996), also has highly conserved PBS1 (77% similarity) and PBS2 (100% similarity) domains. The integrity of both PBS1 and PBS2 on the carboxyl terminus of FAK was found to be essential for paxillin binding and, importantly, for FAK focal adhesion localization. Furthermore, microinjection of FAK constructs containing single point mutations in the PBS domains that eliminated the binding of paxillin also failed to target FAK to focal adhesions. These data suggest that FAK does not target, but is targeted by paxillin to focal adhesions (Tachibana et al., 1995). Also, a report examining fibroblasts from FAK-deficient mice revealed paxillin

*Figure 8.* Truncation mutagenesis localization of the paxillin focal adhesion targeting motif to LIM3. CHO.K1 fibroblasts were transfected with chicken paxillin constructs followed by indirect immunofluorescence analysis using a chicken-specific paxillin polyclonal antiserum (*A*, *C*, *E*, and *G*) and rhodamine-phalloidin to decorate actin stress fibers (*B*, *D*, *F*, and *H*). Paxillin 54-559 (*A*) and 54-LIM3 (*C*) efficiently localized to focal adhesions. However, cells transfected with paxillin 54-LIM2 (*E*) or 54-LIM1 (*G*) showed a cytoplasmic distribution for the chicken paxillin, suggesting a loss of the paxillin focal adhesion targeting motif. Importantly, 54-LIM2 and 54-LIM1 contain intact vinculin- and FAK-binding domains yet lack the capacity to target to focal adhesions. Bar, 5  $\mu$ m.

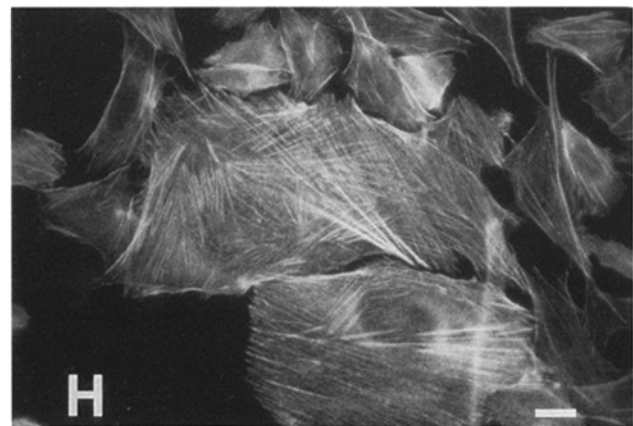
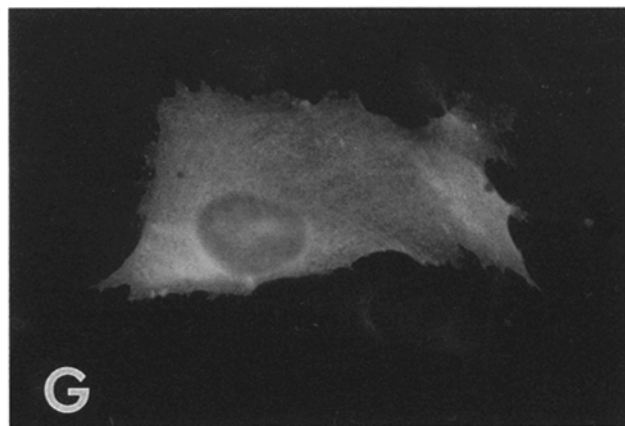
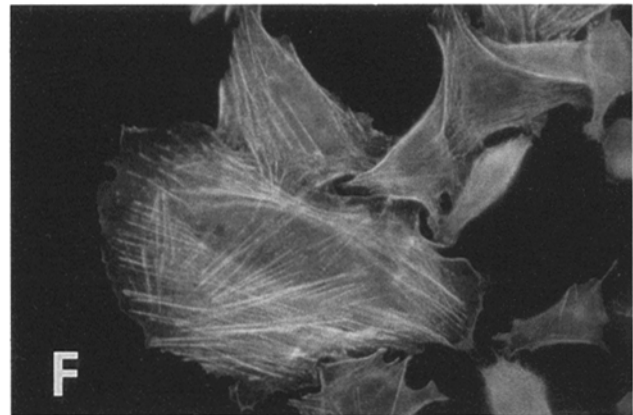
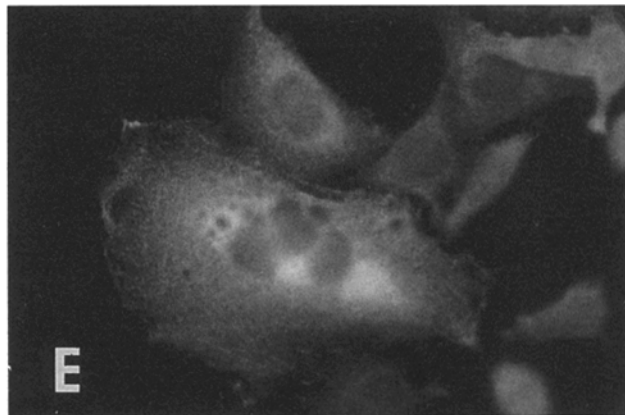
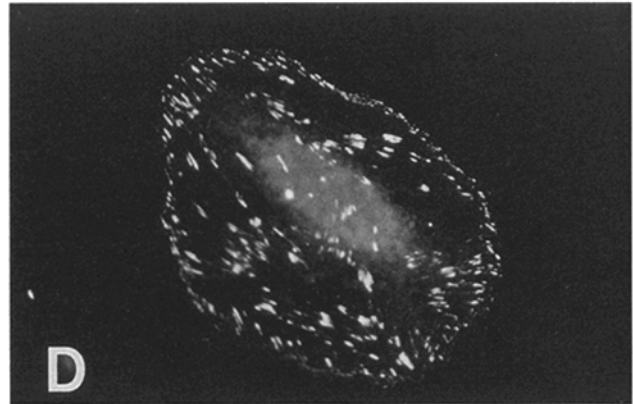
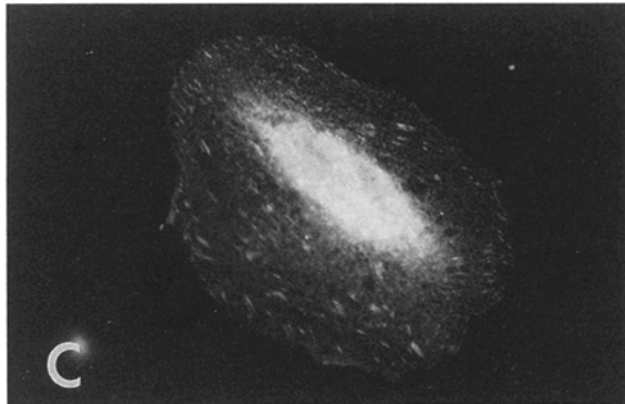
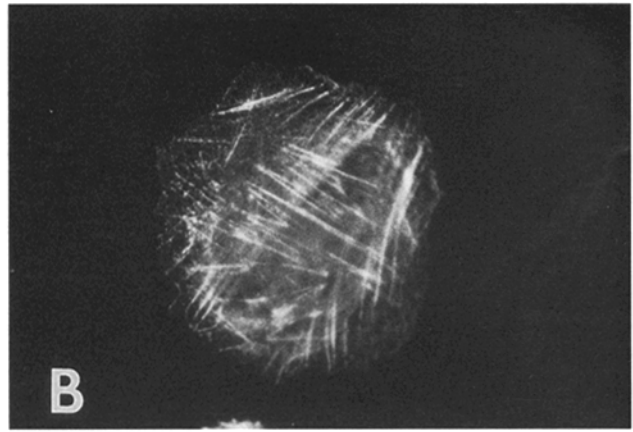
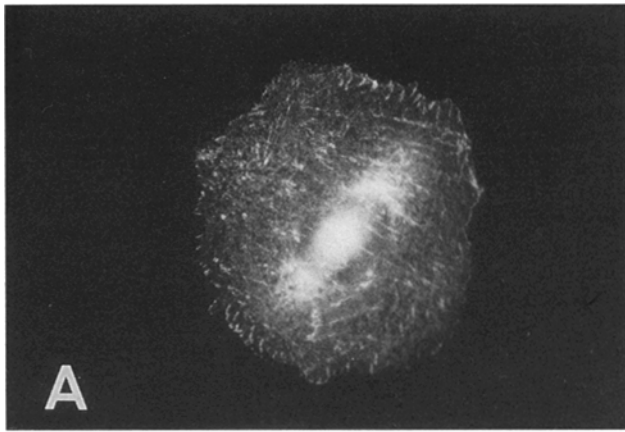


Table III.

## Delineation of the Paxillin Focal Adhesion Targeting Motif

Paxillin Schematic	Construct	Focal Adhesion Localization
	1-559 wild-type	++++
	dl 134-167	++++
	D146N/R147Q	++++
	E151Q	++++
	1-313	-
	168-559	+++
	54-559	++++
	54-LIM3	++++
	54-LIM2	-
	54-LIM1	-
	LIM1 H349I/C352A	++++
	LIM2 H405I/C411A	++
	LIM3 C467A/C470A	-
	dl LIM3 (444-494)	-

tyrosine phosphorylation, an increased number of focal adhesions, and decreased cell motility (Ilić et al., 1995). Unfortunately, the capacity of paxillin to target to focal adhesions was not addressed. Our demonstration of a single binding site for vinculin and two sites for FAK on paxillin correlates with the contiguous nature of PBS sequences on vinculin and the separation of the PBS motifs on FAK. Additionally, our transfection studies demonstrate that the FAK-binding domains within the amino-terminal half of paxillin are insufficient and unnecessary for paxillin focal adhesion localization. When the FAK truncation and deletion mutagenesis study by Hildebrand et al. (1995) is examined in the context of the PBS terminology of Tachibana et al. (1995), it is apparent that the presence of both PBS1 and PBS2, as well as the intervening sequences, is essential for both paxillin binding and FAK focal adhesion localization. On the basis of the local-

ization of the binding of vinculin and FAK to regions of paxillin containing a 13-amino acid LD motif, it is likely that these sequences represent the regions of interaction with the PBS sequences of vinculin and FAK. We are currently examining in more detail the interaction between the individual LD repeats of paxillin and the PBS sequences of vinculin, FAK, and related proteins.

The determination that paxillin localizes to focal adhesions independent of interactions with vinculin and/or FAK (Figs. 8 and 9) raises a question regarding the function of the interaction between paxillin, vinculin, and FAK. Although such binding domains on paxillin are not necessary or sufficient for localization, they are likely to be necessary for stabilization and maintenance of this subcellular distribution. Additionally, these interactions may modulate the capacity of these cytoskeletal proteins to interact with the many other constituents of the focal adhesion (Miyamoto et al., 1995), structurally or enzymatically, thereby modulating the assembly and disassembly of these structures and other signaling pathways associated with integrin function (for review see Juliano and Haskill, 1993). The dynamic nature of paxillin phosphorylation (Turner, 1994) may also affect interactions with associated proteins. Our observation of the paxillin LIM domains, in the absence of the amino terminus, localizing both to focal adhesions and partially extending along the stress fibers, suggests that the focal adhesion localization of paxillin is stabilized through interactions involving the amino-terminal portion of the protein.

Although we have established that LIM3 is the principal determinant of paxillin focal adhesion targeting, our data suggest mutations of LIM2 have an attenuating effect on paxillin focal adhesion targeting. This is consistent with a recent report detailing cooperativity among LIM domains in the subcellular distribution of the LIM protein muscle LIM protein and cysteine-rich protein (Arber and Caroni, 1996). Therefore, a complexity of LIM domain structural interactions affecting the functional role of LIM3 in paxillin focal adhesion targeting is possible. It will be important to determine the identity of the protein responsible for recruiting paxillin to focal adhesions via an interaction with the LIM domains. The LIM motifs of Enigma, a protein of unknown function, are capable of recognizing and binding to regions of proteins containing tyrosine tight turns (NPXY motifs) (Wu and Gill, 1994). Tyrosine tight turns are present on the cytoplasmic tails of  $\beta$  integrins as well as some growth factor receptors (Chen et al., 1990; Backer et al., 1992; Reszka et al., 1992). The directing of paxillin to focal adhesions via the LIM domains is intriguing in view of a recent report detailing the potential interaction of paxillin with the  $\beta_1$  integrin cytoplasmic tail, although it

**Figure 9.** Site-directed and deletion mutagenesis demonstrate that LIM3 is the principal determinant of paxillin focal adhesion localization. CHO.K1 fibroblasts were transfected with chicken paxillin constructs followed by indirect immunofluorescence analysis using a chicken-specific paxillin polyclonal antiserum (A, C, E, and G) and rhodamine-phalloidin to decorate actin stress fibers (B, F, and H) or an mAb to vinculin (D). To further examine a role for the LIM domains of paxillin as the focal adhesion targeting motif, cells were transfected with full-length paxillin constructs in which the structural integrity of the double zinc fingers of individual LIM domains was compromised. Perturbation of LIM1 (H<sup>349</sup>I/C<sup>352</sup>A) (A) or LIM2 (H<sup>405</sup>I/C<sup>411</sup>A) (C) does not abrogate focal adhesion targeting, whereas perturbation of LIM3 (C<sup>467</sup>A/C<sup>470</sup>A) eliminates focal adhesion localization of paxillin (E). Furthermore, deletion of LIM3 (444-494) from the full-length molecule eliminates focal adhesion targeting (G). This demonstrates a role for LIM3 as the principal determinant of paxillin focal adhesion localization. Bar, 5  $\mu$ m.



was not confirmed that this interaction is direct (Schaller et al., 1995). Although paxillin is capable of interacting with tyrosine-containing tight turns (Wu, R.-Y., G.N. Gill, M.C. Brown, and C.E. Turner, unpublished observations), we have been unable, in preliminary experiments, to detect interactions between the four tandem LIM domains of paxillin and the  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ,  $\beta_5$ , or  $\beta_8$  integrin cytoplasmic tails using the yeast two-hybrid system (O'Toole, T.E., M.C. Brown, and C.E. Turner, unpublished observations). The capacity of paxillin to interact with zyxin (Crawford et al., 1992) and other LIM proteins also is currently under examination.

A thorough molecular examination, using tools such as we have defined in this study, will be required to determine the temporal and spatial function of the meshwork of cytoskeletal interactions in regulating these contact sites, and, consequently, the diverse cellular phenomena associated with cell adhesion.

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## References

- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Arber, S., and P. Caroni. 1996. Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. *Genes & Dev.* 10:289-300.
- Avraham, S., R. London, Y. Fu, S. Ota, D. Hiregowdara, J. Li, S. Jiang, L.M. Pasztor, R.A. White, J.E. Groopman, and H. Avraham. 1995. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem.* 270:27742-27751.
- Backer, J.M., S.E. Shoelson, M.A. Weiss, Q.X. Hua, R.B. Cheatham, E. Haring, D.C. Cahill, and M.F. White. 1992. The insulin receptor juxtamembrane region contains two independent tyrosine/ $\beta$ -turn internalization signals. *J. Cell Biol.* 118:831-839.
- Belkin, A.M., O.I. Ornatsky, M.A. Glukhova, and V.E. Koteliensky. 1988. Immunolocalization of meta-vinculin in human smooth and cardiac muscles. *J. Cell Biol.* 107:545-553.
- Bellis, S.L., J.T. Miller, and C.E. Turner. 1995. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J. Biol. Chem.* 270:17437-17441.
- Birge, R.B., J.E. Fajardo, C. Reichman, S.E. Shoelson, Z. Songyang, L.C. Cantley, and H. Hanafusa. 1993. Identification and characterization of a high affinity interaction between v-crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. *Mol. Cell Biol.* 13:4648-4656.
- Burridge, K. 1986. Substrate adhesions in normal and transformed fibroblasts: organization and regulation of cytoskeletal, membrane and extracellular matrix components at focal contacts. *Cancer Rev.* 4:18-78.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487-525.
- Burridge, K., C.E. Turner, and L.H. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125<sup>FAK</sup> accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119:893-903.
- Chen, W.-J., J.L. Goldstein, and M.S. Brown. 1990. NPXY, a sequence often found in cytoplasmic domains, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116-3123.
- Clark, J.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science (Wash. DC)*. 268:233-239.
- Coll, J.-L., A. Ben-Ze'ev, R.M. Ezzell, J.L. Rodríguez Fernández, H. Baribault, R.G. Oshima, and E.D. Adamson. 1995. Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. *Proc. Natl. Acad. Sci. USA*. 92:9161-9165.
- Crawford, A.W., J.W. Michelsen, and M.C. Beckerle. 1992. An interaction between zyxin and  $\alpha$ -actinin. *J. Cell Biol.* 116:1381-1393.
- Fernández, J.L., B. Geiger, D. Salomon, and A. Ben-Ze'ev. 1993. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. *J. Cell Biol.* 122:1285-1294.
- Feuerstein, R., X. Wang, D. Song, N.E. Cooke, and S.A. Liebhaber. 1994. The LIM/double zinc-finger motif functions as a protein dimerization domain. *Proc. Natl. Acad. Sci. USA*. 91:10655-10659.
- Guan, J.-L., and D. Shalloway. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature (Lond.)*. 358:690-692.
- Hanks, S.K., M.B. Calalb, M.C. Harper, and S.K. Patel. 1992. Focal adhesion-protein kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. USA*. 89:8487-8491.
- Hemmings, L., S.T. Barry, and D.R. Critchley. 1995. Cell-matrix adhesion: structure and regulation. *Biochem. Soc. Trans.* 23:619-626.
- Hildebrand, J.D., M.D. Schaller, and J.T. Parsons. 1995. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Mol. Biol. Cell.* 6:637-647.
- Hynes, R.O. 1992. Integrin: versatility, modulation, and signaling in cell adhesion. *Cell*. 69:11-25.
- Ilić, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, T. Yamamoto, and S. Alzawa. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature (Lond.)*. 377:539-544.
- Juliano, R.L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577-585.
- Kornberg, L., H.S. Earp, J.T. Parsons, M. Schaller, and R.L. Juliano. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439-23442.
- Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA*. 82:488-492.
- Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J.M. Musacchio, G.D. Plowman, B. Rudy, and J. Schlessinger. 1995. Protein tyrosine kinase PYK2 involved in  $Ca^{2+}$ -induced regulation of ion channel and MAP kinase functions. *Nature (Lond.)*. 376:737-745.
- Li, J., H. Avraham, R.A. Rogers, S. Raja, and S. Avraham. 1996. Characterization of RAFTK, a novel focal adhesion kinase, and its integrin-dependent phosphorylation and activation in megakaryocytes. *Blood*. 88:417-428.
- Lo, S.H., and L.B. Chen. 1994. Focal adhesion as a signal transduction organelle. *Cancer Metastasis Rev.* 13:9-24.
- Luna, E.J., and A.L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. *Science (Wash. DC)*. 258:955-964.
- Miyamoto, S., J. Teramoto, O.A. Coso, J.S. Gutkind, P.D. Burbelo, S.K. Akiyama, and K.M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131:791-805.
- Okano, I., J. Hiraoki, H. Otera, K. Ninoue, K. Ohashi, S. Iwashita, M. Hirai, and K. Mizuno. 1995. Identification and characterization of a novel family of serine/threonine kinase containing N-terminal LIM motifs. *J. Biol. Chem.* 270:31321-31330.
- O'Toole, T.E., J. Ylanne, and B.M. Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the  $\beta$  subunit cytoplasmic domain. *J. Biol. Chem.* 270:8553-8558.
- Rabbitts, T.H., and T. Boehm. 1990. LIM domains. *Nature (Lond.)*. 346:418.
- Ren, R., B.J. Mayer, P. Cichetti, and D. Baltimore. 1993. Identification of a ten-amino acid proline-rich SH3 binding site. *Science (Wash. DC)*. 259:1157-1161.
- Reszka, A.A., Y. Hayashi, and A.F. Horwitz. 1992. Identification of amino acid sequences in the integrin  $\beta_1$  cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321-1330.
- Sadler, I., A.W. Crawford, J.W. Michelsen, and M.C. Beckerle. 1992. Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* 119:1573-1587.
- Samuels, M., R.M. Ezzell, T.J. Cardozo, D.R. Critchley, J.-L. Coll, and E.D. Adamson. 1993. Expression of chicken vinculin complements the adhesion-defective phenotype of a mutant mouse F9 embryonal carcinoma cell. *J. Cell Biol.* 121:909-921.
- Sánchez-García, I., and T.H. Rabbitts. 1994. The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet.* 10:315-320.
- Sasaki, H., K. Nagura, M. Ishino, H. Tobioka, K. Kotani, and T. Sasaki. 1995. Cloning and characterization of cell adhesion kinase  $\beta$ , a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J. Biol. Chem.* 270:21206-21219.
- Schaller, M.D., and J.T. Parsons. 1995. pp125<sup>FAK</sup>-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for crk. *Mol. Cell Biol.* 15:2635-2645.
- Schaller, M.D., C.A. Borgman, and J.T. Parsons. 1993. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125<sup>FAK</sup>. *Mol. Cell Biol.* 13:785-791.
- Schaller, M.D., C.A. Otey, J.D. Hildebrand, and J.T. Parsons. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking  $\beta$  integrin cytoplasmic domains. *J. Cell Biol.* 130:1181-1187.
- Shibanuma, M., J.-I. Mashimo, T. Kuroki, and K. Nose. 1994. Characterization of the TGF $\beta$ 1-inducible *hic-5* gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. *J. Biol. Chem.* 269:26767-26774.
- Tachibana, K., T. Sato, N. D'Avirro, and C. Morimoto. 1995. Direct association of pp125<sup>FAK</sup> with paxillin, the focal adhesion-targeting mechanism of pp125<sup>FAK</sup>. *J. Exp. Med.* 182:1089-1100.

- Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Turner, C.E. 1994. Paxillin: a cytoskeletal target for tyrosine kinases. *Bioessays.* 16:47-52.
- Turner, C.E., and K. Burridge. 1990. Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol.* 3:849-853.
- Turner, C.E., and J.T. Miller. 1994. Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125<sup>FAK</sup> binding region. *J. Cell Sci.* 107:1583-1591.
- Turner, C.E., J.R. Glenney, and K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111:1059-1068.
- Turner, C.E., M.D. Schaller, and J.T. Parsons. 1993. Tyrosine phosphorylation of the focal adhesion kinase pp125<sup>FAK</sup> during development: relation to paxillin. *J. Cell Sci.* 105:637-645.
- Turner, C.E., K.M. Pietras, D.S. Taylor, and C.J. Molloy. 1995. Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells. *J. Cell Sci.* 108:333-342.
- Volberg, T., B. Geiger, Z. Kam, R. Pankov, I. Simcha, H. Sabanay, J.-L. Coll, E. Adamson, and A. Ben-Ze'ev. 1995. Focal adhesion formation by F9 embryonal carcinoma cells after vinculin gene disruption. *J. Cell Sci.* 108:2253-2260.
- Weng, Z., J.A. Taylor, C.E. Turner, J.S. Brugge, and C. Seidel-Dugan. 1993. Detection of src-SH3 binding proteins, including paxillin, in normal and v-src-transformed BALBc/3T3 cells. *J. Biol. Chem.* 268:14956-14963.
- Wood, C.K., C.E. Turner, P. Jackson, and D.R. Critchley. 1994. Characterisation of the paxillin-binding site and the C-terminal focal adhesion targeting sequence in vinculin. *J. Cell Sci.* 107:709-717.
- Wu, R.-Y., and G.N. Gill. 1994. LIM domain recognition of a tyrosine-containing tight turn. *J. Biol. Chem.* 269:25085-25090.