Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125Fak-binding region

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SUMMARY

Paxillin is a cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix. Extensive tyrosine phosphorylation of this protein occurs during integrin-mediated cell adhesion, embryonic development, fibroblast transformation and following stimulation of cells by mitogens that operate through the family of seven membrane-spanning G-protein-coupled receptors. Paxillin binds in vitro to the focal adhesion protein vinculin as well as to the SH3 domain of c-src and, when tyrosine phosphorylated, to the SH2 domain of v-crk. Here, we report the complementary DNA, and derived amino acid sequence, that codes for approximately 90% of the paxillin protein. We have identified a region in the amino-terminal half of the protein that supports the binding of both vinculin and the focal adhesion tyrosine kinase, pp125Fak. Although there is no significant overall homology with other identified proteins, the carboxyl third of paxillin contains one LIM domain and three LIM-like sequences. The LIM motif is common to a number of transcription factors and to two other focal adhesion proteins, zyxin and cysteine-rich protein. In addition to several potential tyrosine phosphorylation sites there are five tyrosine-containing sequences that conform to SH2-binding motifs. The protein also contains a short proline-rich region indicative of a SH3-binding domain. Taken together, these data suggest that paxillin is a unique cytoskeletal protein capable of interaction with a variety of intracellular signaling, and structural, molecules important in growth control and the regulation of cytoskeletal organization. The homology with zyxin and cysteine-rich protein defines a novel family of LIM-containing molecules associated with sites of actin-membrane attachment.

Key words: cytoskeleton, signalling, Src homology, SH2, SH3, tyrosine phosphorylation

INTRODUCTION

Paxillin (Turner et al., 1990; Turner, 1994) is a 68 kDa protein that, in cultured cells, contributes to a complex of cytoskeletal proteins serving to link the actin cytoskeleton to the plasma membrane at sites of cell adhesion to the extracellular matrix, known as focal adhesions (Burrage et al., 1988; Turner and Burrage, 1991). Integrin molecules (Hynes, 1992) provide the transmembrane connection between the cytoskeletal components and the extracellular matrix in these structures. Paxillin is similarly recruited to actin-membrane attachment sites in vivo, including the dense plaques of smooth muscle and the myotendinous junctions of skeletal muscle (Turner et al., 1991). Paxillin binds in vitro to the carboxyl terminus of vinculin (Turner et al., 1990; Wood et al., 1994), a major structural component of focal adhesions. A similar association with vinculin in vivo could account for paxillin’s localization to focal adhesions. However, additional interactions in vitro between paxillin and both the SH3 domain of pp60c-src (Weng et al., 1993) and the SH2 domain of v-crk (Birge et al., 1993) have been reported, thus implicating other potential mechanisms of recruitment of paxillin to the plasma membrane.

Paxillin is a major target for tyrosine kinases during various cellular events associated with cell adhesion and growth control. It was first identified as one of many substrates for pp60c-src in Rous sarcoma virus (RSV)-transformed chick embryo fibroblasts (CEFs) (Glenney and Zokas, 1989). Subsequently, it has been reported to be phosphorylated on tyrosine residues in a developmentally regulated manner in the chick embryo (Turner, 1991; Turner et al., 1993), during integrin-mediated fibroblast adhesion to extracellular matrix in cultured fibroblasts (Burrage et al., 1992), and in response to a variety of mitogens including the neuropeptides bombesin, vasopressin and endothelin (Zachary et al., 1993), and angiotensin II and thrombin (C. E. Turner, K. M. Peitras, D. S. Taylor and C. J. Molloy, unpublished observations). Tyrosine phosphorylation of paxillin is invariably accompanied by a similar increase in the tyrosine phosphorylation of
the focal adhesion tyrosine kinase pp125Fak (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Schaller et al., 1992; Schaller and Parsons, 1993; Romer et al., 1993; Zachary et al., 1993; Turner, 1994), suggesting that the activity of pp125Fak and paxillin tyrosine phosphorylation are closely coupled. Indeed, the tyrosine phosphorylation of paxillin by a pp125Fak immunoprecipitate in vitro (Turner et al., 1993) suggests that paxillin is a substrate for pp125Fak in vivo.

In each of the examples described above, with the exception of sarcoma virus transformation, tyrosine phosphorylation of paxillin is accompanied by cytoskeletal reorganization that results in focal adhesion and actin stress fiber assembly (Burridge et al., 1992; Turner, 1994). RSV transformation, in contrast, causes a disruption of these structures reflecting a perturbation of normal signaling processes (Burridge, 1986; Glenney, 1992). It is possible therefore, that the tyrosine phosphorylation of paxillin provides a mechanism for regulating its interaction both with signaling molecules and with other cytoskeletal-associated proteins. The formation of such signaling complexes and the accompanying structural reorganization of the cytoskeleton may contribute to the regulation of normal cellular functions such as cell adhesion, gene expression and growth. The cloning and sequencing of paxillin cDNAs described in this report and their expression as bacterial fusion proteins expands our understanding of the role of paxillin in these cellular events.

**MATERIALS AND METHODS**

**Cloning strategy**

Screening λgt11 expression libraries with the monoclonal anti-paxillin antibody (165) (Glenney and Zokas, 1989; Turner et al., 1990) failed to produce any positive clones. Therefore, the following strategy was employed. Paxillin was purified from adult chicken gizzard as previously described (Turner et al., 1990), electrophoresed and transferred to nitrocellulose. The paxillin (68 kDa) band was excised and processed to obtain the internal peptide sequences listed in Table 1 (peptide sequencing was performed at either the Harvard Microsequencing Facility or Glaxo (Bill Birkhart), Research Triangle Park, NC). Degenerate oligonucleotide primers were designed to the probable sequence encoding residues 2-10 (forward oligonucleotide (peptide 8, Table 1) and residues 20-27 (reverse oligonucleotide 5'-TGCCATCICITCTCAAAIATT'-3) of the least degenerate peptide (peptide 7). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on embryonic chicken gizzard RNA using standard procedures (Sambrook et al., 1989). A single PCR product with a predicted size of approximately 80 bp was isolated, subcloned into the TA vector pc2000 (Invitrogen) and sequenced. The central portion of this cDNA provided the non-degenerate sequence encoding for the central portion of peptide 7 (residues 11-19). A forward oligonucleotide primer complementary to this region (5'-ACCCACTGCACAGGAGAATTGGAACGG-3') was utilized in a 3' RACE reaction (Innis et al., 1990) using embryonic chicken gizzard total RNA as the template. Oligo-dT5'-coupled to an oligonucleotide linker was used as the reverse primer for the RT reaction and the linker portion plus the paxillin-specific primer were employed in the subsequent PCR. A 500 bp fragment was generated, subcloned into the TA vector and sequenced. This cDNA contained sequence encoding for an additional paxillin peptide (peptide 8, Table 1) and was used to screen an embryonic chicken λgt11 cDNA library (Clonetech). Two clones (designated 22 (1.8 kb) and 10 (2 kb) were isolated, subcloned into pBluescript and sequenced. The longest clone (clone 10) (residues 110-2063 in Fig.1) contains coding regions corresponding to the peptide sequences 2-10 (Table 1). In addition, it contains 396 bp of 3' untranslated sequence. We have not isolated cDNA clones from the libraries containing the polyadenylation sequence. Although there was an overlap of approximately 140 bp between clone 10 and the 500 bp PCR product, the 3' ends were divergent. While this may be the result of alternative splicing, the PCR product was probably generated from unprocessed mRNA, since the point of divergence occurs at a potential RNA 5' splice junction (data not shown).

Clones 10 and 22 terminated within 4 bp of each other at their 5' end. Screening of a random primed chicken embryo fibroblast (CEF) cDNA library (generously provided by Dr W. Zimmer, University of Alabama) with clone 10 resulted in additional clones that were all truncated in the same 5' region, suggesting that secondary structure had prevented successful reverse transcription through this region during library construction. Therefore, a chicken genomic library in the EMBL 3 vector (kindly provided by Dr J. T. Parsons, University of Virginia) was screened with clone 10. Positive plaques were isolated and SacI-digested phage DNA preparations were screened by Southern blotting (Sambrook et al., 1989), both with an oligonucleotide corresponding to the 5' end of clone 10 and with a degenerate oligonucleotide to the remaining paxillin peptide (peptide 1), not yet accounted for. A 3 kb SacI fragment that hybridized with both probes was subcloned into pBluescript and sequenced using paxillin-specific primers. An additional 110 nucleotides were obtained in this manner before encountering the first 5' intron/exon boundary. The complete sequence encoding peptide 1 was contained within this exon fragment. In spite of repeated attempts we have been unable to obtain the final piece of cDNA encoding the 5' region of the paxillin gene. In all cases dioxygenylcide chain termination sequencing of double-stranded DNA was performed on both strands using Sequenase (US Biochemical Corp.). Sequence analysis and alignment was performed using the University of Wisconsin GCG (UWGCG) software package (Devereux et al., 1984).

**Northern blot analysis**

Total RNA was extracted from embryonic day 16 chicken gizzard (smooth muscle tissue) using standard protocols (Sambrook et al., 1989). Total RNA (10-20 µg) was electrophoresed overnight in 1% formaldehydeagarose gels. Fractionated RNA was transferred to nylon membrane (Bio-Rad) and hybridized with random-primed 32P-labeled DNA probe (clone 10). The hybridization and washing conditions were as described (Sambrook et al., 1989). A 1.6 kb vinculin cDNA probe corresponding to the protein's amino-terminal region was generated by PCR (oligonucleotides kindly provided by Brian Kay, UNC-Chapel Hill) for use in verifying the quality of the mRNA.

**Glutathione S-transferase (GST) fusion proteins**

Selected fragments of paxillin cDNA were generated by PCR using

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**Table 1. Peptide sequences derived from purified paxillin**

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SSSASVPRDGLSSPSPRASEEEHVYSFPNK</td>
<td>Sequence 1 from adult chicken gizzard.</td>
</tr>
<tr>
<td>DEVSRSPSLPNVTGPHYVI</td>
<td>Sequence 2 from adult chicken gizzard.</td>
</tr>
<tr>
<td>RD</td>
<td>Sequence 3 from both preparations.</td>
</tr>
<tr>
<td>SS</td>
<td>Sequence 4 from both preparations.</td>
</tr>
<tr>
<td>DYFDMFAPK</td>
<td>Sequence 5 from both preparations.</td>
</tr>
<tr>
<td>SGSSGIRDVRSPVEILLDLESSV</td>
<td>Sequence 6 from both preparations.</td>
</tr>
<tr>
<td>TWHKPEFVCTCHOIEIGSRNIFERDGQ</td>
<td>Sequence 7 from both preparations.</td>
</tr>
<tr>
<td>DYNLNPSCRYCYN</td>
<td>Sequence 8 from both preparations.</td>
</tr>
<tr>
<td>VVTALDRWNHFEPFYACQCGVFPPEG</td>
<td>Sequence 9 from both preparations.</td>
</tr>
<tr>
<td>DYFDMAPK</td>
<td>Sequence 10 from both preparations.</td>
</tr>
</tbody>
</table>

These peptides are ordered as they appear in the sequence in Fig. 1 starting with the most amino-terminal. Sequences were derived from two separate protein preparations. Sequence 3 was obtained from both preparations. Minor differences between the peptide sequences and those derived from the cDNA are underlined in this table.
Cloning of paxillin oligonucleotides containing 5′ BamHI or 3′ EcoRI restriction sites and subcloned in-frame into BamHI/EcoRI-digested pGEX-2T plasmid expression vector (Pharmacia). Correct in-frame incorporation was verified by sequencing with pGEX-specific oligonucleotides. To express the glutathione S-transferase (GST)-paxillin fusion proteins, Escherichia coli (DH5α) transformed with the appropriate pGEX-paxillin construct were grown overnight, diluted 1:10 and grown for 1.5 hours. Protein expression was induced for 3 hours by the addition of 0.1-0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 10,000 g for 10 minutes and lysed directly into SDS-PAGE sample buffer for gel analysis. Alternatively, the fusion protein was purified by first lysing the bacteria in Tris-buffered saline, pH 7.4 (TBS), containing 2 mg/ml lysozyme, 0.1% β-mercaptoethanol and protease inhibitors for 30 minutes at room temperature. Triton X-100 (TX-100) was added to 1% and the cells were incubated on ice for 10 minutes. Bacterial cell wall debris was removed by centrifugation at 10,000 g for 15 minutes. Protein expression was induced for 3 hours by the addition of 0.1-0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 10,000 g for 10 minutes and lysed directly into SDS-PAGE sample buffer for gel analysis. Alternatively, the fusion protein was purified by first lysing the bacteria in Tris-buffered saline, pH 7.4 (TBS), containing 2 mg/ml lysozyme, 0.1% β-mercaptoethanol and protease inhibitors for 30 minutes at room temperature. Triton X-100 (TX-100) was added to 1% and the cells were incubated on ice for 10 minutes. Bacterial cell wall debris was removed by centrifugation at 10,000 g for 15 minutes. Protein expression was recovered from the supernatant by incubation with glutathione-agarose (Pharmacia) according to the manufacturer’s instructions. Fusion protein was eluted from the beads by incubation with 20 mM glutathione in 50 mM Tris-HCl, pH 8.0, 0.1% β-mercaptoethanol, and then dialyzed into the appropriate buffer. Fusion proteins were then purified by gel electrophoresis, transferred to nitrocellulose and injected into rabbits to generate polyclonal antisera.

GST-paxillin binding assays

A GST-paxillin fusion protein spanning amino acids 51-308 (GST-51-308) was generated by PCR using oligonucleotide primers containing 5′ BamHI and 3′ EcoRI restriction sites. The fragment was purified, digested with BamHI/EcoRI, and ligated into BamHI/EcoRI-digested pGEX-2T. Protein expression and purification by binding to glutathione-agarose beads was as described above. For binding assays a lysate of embryonic day 15 chicken gizzard was prepared by homogenizing the tissue in ten volumes of lysis buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1% β-mercaptoethanol, 1% Triton X-100 and protease inhibitors. The lysate was clarified at 100,000 g for 30 minutes. Samples of lysate (1 mg of protein) were incubated with GST-51-308 fusion protein coupled to the glutathione-agarose beads or with GST-agarose for 60 minutes, washed extensively in lysis buffer and boiled directly in SDS-PAGE sample buffer. Following electrophoresis, the proteins were stained with Coomassie Blue or transferred to nitrocellulose and probed with the appropriate antibody.

Immunofluorescence microscopy

Chick embryo fibroblasts were cultured on glass coverslips and

Fig. 1. Nucleotide and derived amino acid sequence of chicken paxillin. Numbering of nucleotides is displayed in the left margin and amino acids on the right. Regions of the deduced amino acid sequence that correspond to the microsequenced paxillin peptides are underlined. The putative SH3-binding, proline-rich, region is boxed (second line). The one LIM and three LIM-like domains, contained within the C-terminal third of the molecule, are also boxed. Tyrosine residues that are potential sites for phosphorylation are marked with an *. Putative SH2-binding motifs are marked by four consecutive * . Six potential sites for phosphorylation by protein kinase C are marked with # . The sequence data has been deposited in the EMBL/GenBank database under accession number L30099.
processed for immunofluorescence microscopy as described previously (Turner et al., 1990). Photographs were taken on a Zeiss Axiophot photomicroscope equipped with epifluorescence illumination using Kodak T-Max 400 film.

**SDS-PAGE, immunoprecipitation and western blotting**
Electrophoresis was performed as described (Laemmli, 1970) using either 10% or 12.5% polyacrylamide gels. Western transfer and blotting, including radiolodination of secondary antibodies, was performed as described previously (Towbin et al., 1979; Turner, 1991). Immunoprecipitations were performed on extracts of adult chicken gizzard as described (Turner, 1991). The immune complexes, harvested by centrifugation following binding to Protein A-Sepharose, were boiled directly in SDS-PAGE sample buffer. Antibodies used in this study were as follows. Paxillin, monoclonal antibody 165 (Turner et al., 1990), a polyclonal antiserum, Pax 2, characterized in this paper. Vinculin, monoclonal antibody, Vin-11-5. Talin, monoclonal antibody 8D4 (a gift from Keith Burridge, UNC). Pp125Fak, polyclonal antibody, BC3 (a gift from Tom Parsons, UVA).

**RESULTS AND DISCUSSION**

**Verification of paxillin cDNA authenticity**
We have isolated a series of overlapping cDNA clones using a combination of PCR, and screening of chicken cDNA and genomic DNA libraries (see Materials and Methods for details). When combined, they represent an open reading frame of 1662 bp encoding for 554 amino acids (61 kDa) (Fig. 1). Based on the mobility of paxillin on SDS-PAGE gels, where it migrates as a 68 kDa protein, this sequence is equivalent to approximately 90% of the paxillin protein.

In addition to the presence of regions encoding all of the peptides derived from internal sequencing of purified paxillin (Table 1, and underlined in Fig. 1), the following experiments were performed to confirm the authenticity of the paxillin sequence. First, a polyclonal antibody was raised against a glutathione S-transferase (GST)-paxillin fusion protein encompassing amino acids 51 to 458 (GST-51-458). The resulting antiserum (Pax 2) was used to probe a Western blot of total chicken gizzard protein (Fig. 2A). The antibody recognized a protein of approximately 68 kDa (Fig. 2A, lane 1) that comigrated with a protein detected by the anti-paxillin monoclonal antibody 165 (lane 1′). In addition, the GST-51-458 antibody, but not the preimmune rabbit serum, immunoprecipitated a 68 kDa protein from a chicken gizzard lysate that was recognized by the paxillin monoclonal antibody in a subsequent western blot (Fig. 2B, lane 2). To confirm further the authenticity of the cDNA, the staining pattern for the GST-51-458 antiserum was determined in fixed, permeabilized chick embryonic fibroblasts (CEF) (Fig. 3). The fusion protein antibody (Fig. 3A and C) co-localized with the monoclonal anti-paxillin antibody (Fig. 3B) in a pattern consistent with the labeling of focal adhesions. This focal adhesion staining pattern was confirmed to be localized to the ends of stress fibers by double-labeling with RITC-phalloidin (Fig. 3C and D).

**Northern blot analysis**
Identical samples of total RNA isolated from embryonic day 16 chicken gizzard were probed in a northern blot with either a vinculin-specific cDNA (Fig. 4, lane 1) or a paxillin-specific cDNA (lane 2). The vinculin probe recognized a message of 6.2 kb corresponding to full-length vinculin mRNA (Price et al., 1987). The paxillin probe hybridized to a single message of approximately 3.5 kb.

**Analysis of paxillin sequence**

**LIM domains**
The paxillin sequence was compared with the Swiss Protein and GenEMBL protein data bases using the FASTA program (UWGCG). The highest level of homology was found to be with another low abundance focal adhesion protein, zyxin (Beckerle, 1986; Sadler et al., 1992). The homology is restricted to cysteine-rich regions, organized into motifs known as LIM domains (Freyd et al., 1990). The consensus sequence for LIM domains is CX2 CX16-23 HX2 CX2 CX2 CX16-21 CX2-3 (C,H,D). Paxillin contains four cysteine-rich regions (boxed in Fig. 1). The third of these domains conforms precisely to the LIM consensus. However, the paxillin sequence differs from the currently accepted LIM motif in domains 1, 2 and 4 by containing a conservative cysteine-histidine substitution at the third cysteine residue. Another similar motif, the RING finger motif (Lovering et al., 1993), has the consensus CX2 CX9-27 CX1-3 HX2 CX2 CX6-47 CX5-C. Accordingly, the paxillin domains 1, 2 and 4 have a conservative cysteine-histidine substitution of the third cysteine when compared with the RING

![Image](image-url)
finger consensus sequence. Also, the final cysteine of the RING domain, invariant in the 27 proteins possessing this motif (Lovering et al., 1993), is replaced by an aspartic acid residue in paxillin domains 1 and 2. In view of these apparently minor differences it is unclear at present whether the first, second and fourth cysteine-rich domains of paxillin should be classified as LIM or RING domains or whether they represent a novel type of structural domain. For simplicity we will continue to describe them collectively as LIM domains in this paper, since one of the domains conforms precisely to the LIM motif. Furthermore, all four of the paxillin LIM domains span 51 amino acids and share significant homology with each other. This ranges from a 51% identity between the first and second LIM domains to 38% identity between LIM domains two and four. Each pair of LIM domains is separated by eight amino acids. This high level of homology, which includes the presence of an aromatic residue prior to the first invariant histidine residue, provides additional support for the four domains being part of the same family. The LIM domains of paxillin are aligned in Fig. 5 with the three LIM domains of zyxin. The greatest homology between the LIM domains of paxillin and zyxin is the 33% identity of paxillin’s fourth LIM domain with the second of zyxin. Paxillin and zyxin are unique with respect to the positioning of their LIM domains within the carboxyl third of each protein. In other multi-domain proteins the LIM domains are in the amino half of the molecule (Sadler et al., 1992).

The acronym, LIM, is derived from three homeodomain proteins, Lin-11 (Freyd et al., 1990), Isl-1 (Karlsson et al., 1990) and Mec-3 (Freyd et al., 1990), which each contain two of these structural elements. The precise function of the LIM domains is unclear, although they are found in a number of proteins important in cell lineage determination and pattern formation during development. For example, Lin-11 and Mec-3 are involved in determination of vulval cell lineage (Ferguson et al., 1987) and touch receptor neuron differentiation (Way and Chalfie, 1988), respectively, in Caenorhabditis elegans. We have previously demonstrated that the expression of paxillin is both tissue-specific and developmentally regulated. (Turner, 1991; Turner et al., 1993). Therefore,
it is possible that paxillin may also regulate aspects of cellular development and differentiation, perhaps involving modulation of cell attachment to the extracellular matrix.

Structural predictions indicate that each LIM domain could bind two Zn$^{2+}$ through interactions with the conserved cysteine and histidine residues, thereby forming two zinc fingers (Liebhaber et al., 1990). This has been confirmed for zyxin (Sadler et al., 1992). In vitro studies have implicated a direct binding between the LIM domains of zyxin and another LIM-containing protein, cysteine-rich protein (cCRP) (Sadler et al., 1992). In general therefore, protein-protein interactions between LIM domains may serve to regulate both the intracellular location and the activity of LIM proteins. In this regard, paxillin, zyxin and cCRP co-localize to the cytoplasmic face of focal adhesions. We are currently exploring the possibility that paxillin interacts directly with one or both of these proteins. The homeodomain-containing LIM proteins are presumed to function as transcription factors (Freyd et al., 1990; Karlsson et al., 1990). We have no evidence for a nuclear localization of paxillin or zyxin, suggesting, based on their subcellular localization to focal adhesions, that these proteins constitute a distinct family of cytoplasmic LIM proteins. Nevertheless, these cytoskeleton-associated LIM proteins could potentially interface with LIM-containing transcription factors and thereby target them to signaling complexes positioned close to the plasma membrane, where they would be optimally positioned to respond to external stimuli.

**SH3 and SH2 binding domains**

The amino-terminal half of the paxillin sequence does not contain any extensive regions of homology with proteins in the databases. However, a number of short motifs implicated in binding to signaling molecules were identified within the paxillin sequence. Firstly, a proline-rich region (PPPVPPPPSS) encompassing amino acids 43 to 52 (boxed in Fig. 1) conforms closely to the consensus for the abl SH3 (src homology 3)-binding domain, XPXXPPPψXP (where X is any amino acid and ψ represents a hydrophobic residue (Ren et al., 1993)). Additional SH3 binding motifs have been identified; for example, in the GRB2 binding protein Sos1 (Egan et al., 1993) and p59$^{fyn}$ (Kanteti et al., 1993). These sequences all contain a proline-rich region but differ significantly from each other and from the consensus sequence listed above. Therefore, it is likely, as is the case with SH2 domains (see below), that there are groups of SH3 domains with different preferred recognition sequences. The precise function of SH3 domains is unknown. In addition to their presence in non-receptor tyrosine kinases, where they participate in the regulation of the kinase and the binding of specific substrate molecules (Koch et al., 1991; Pawson and Gish, 1992), SH3 domains are also found in a collection of cytoskeletal and membrane-associated proteins that have no identified catalytic activity; for example, spectrin and myosin 1 (Drubin et al., 1990; Mayer and Baltimore, 1993). It has been suggested therefore, that protein-protein interactions between SH3 domains and their target sequences function to direct signaling and effector molecules to precise subcellular locations. In support of this hypothesis, microinjection studies have demonstrated that the SH3 domains alone of the signaling molecules phospholipase Cγ

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**Fig. 4.** Northern blot analysis. Total embryonic chicken gizzard RNA was electrophoresed on a formaldehyde/agarose gel prior to transfer to a nylon membrane. Duplicate samples were probed with either a vinculin cDNA probe (lane 1) that identified the expected 6.2 kb message, or with a paxillin-specific probe (lane 2). A single transcript of 3.5 kb was detected.

**Fig. 5.** Alignment of paxillin LIM and ‘LIM-like’ domains with those of zyxin. Each LIM domain is numbered according to its position relative to the amino terminus. The absolutely conserved amino acids are boxed. The paxillin LIM domains 1, 2 and 4 differ from this consensus by having a conservative cysteine to histidine substitution at the third cysteine residue of the consensus sequence. An invariant aromatic residue is marked with a ψ. It is predicted that the first four Cys/His residues bind one Zn$^{2+}$ and the second three plus either a Cys/His or Asp bind another, resulting in two ‘zinc fingers’, the length of which being determined by the number of intervening amino acids. In this regard all four paxillin LIM domains contain the same number of residues while those of zyxin vary in size.
function to separate distinct structural domains within proteins; for example, vinculin (Coutu and Craig, 1988).

Many tyrosine kinases contain SH2 (src homology 2) domains. Phosphorylation of tyrosine residues of target molecules induces binding to these domains, thereby regulating the recruitment of specific effector molecules to protein complexes involved in tyrosine kinase-mediated signal transduction pathways (Koch et al., 1991; Pawson and Gish, 1992). An extensive study of SH2 binding motifs has identified groups of tyrosine-containing tetrapeptides that exhibit selectivity of binding to particular SH2 domains (Songyang et al., 1993). The deduced primary sequence of paxillin contains a number of likely tyrosine phosphorylation sites (marked with * in Fig. 1). Included in this group are five tyrosine residues (Y_{35}, Y_{113}, Y_{177}, Y_{431} and Y_{485}) that are followed by short tripeptides demonstrating a high level of homology to predicted SH2 binding motifs (Songyang et al., 1993) for src family members (Y_{35} and Y_{485}), v-crk (Y_{113}), phospholipase Cγ (Y_{177}) and p85 (Y_{431}). Interestingly, residue Y_{35} is just amino-terminal to paxillin’s putative SH3 binding domain and therefore these two regions may operate together, forming a stable association with src (or src-related kinase) that could be regulated by the phosphorylation state of the tyrosine residue. It is likely that the sequence Y_{113} SFP is responsible for the interaction in vitro between tyrosine-phosphorylated paxillin and v-crk (Birge et al., 1993). Although v-crk does not contain a kinase domain it induces transformation in CT10-CEFs by elevating tyrosine phosphorylation levels of a number of proteins, including paxillin (Birge et al., 1993). Therefore, in v-crk-transformed cells, the interaction between v-crk and tyrosine-phosphorylated paxillin may disrupt cellular processes and cytoskeletal organization dependent upon the dephosphorylation of paxillin or compete with other SH2-containing proteins for binding to paxillin. For example, the actin-binding protein tensin (Davis et al., 1991) contains an SH2 domain (the binding motif for this domain has not yet been determined). In addition, this protein localizes to focal adhesions. A tyrosine phosphorylation-dependent interaction between paxillin and the SH2 domain of tensin could contribute to the formation of a nucleating site for subsequent actin-cytoskeleton assembly and might present a key target site for v-crk activity.

In addition to the presence of tyrosine phosphorylation sites, paxillin contains six target sites for phosphorylation by the serine/threonine kinase, protein kinase C (PKC). These are marked with a # in Fig. 1. The potential physiological role of paxillin phosphorylation by PKC is unknown. However, it has been shown that activation of protein kinase C is required, in addition to tyrosine kinase activation, for focal adhesion formation (Birge et al., 1993; Woods and Couchman,

![Fig. 6. Paxillin supports binding of vinculin and pp125Fak. A GST-paxillin fusion protein spanning residues 51-308 (GST-51-308) was incubated with a lysate of embryonic day 15 gizzard. The fusion protein and associated proteins were collected by binding to glutathione-agarose beads (lanes 3). Similar incubations were performed with GST (29 kDa) alone (lanes 2). These samples were co-electrophoresed with a sample of total embryonic gizzard lysate (lanes 1) and either stained with Coomassie Blue (CB) or blotted with antibodies to vinculin (VN), talin (TN) or pp125 Fak (FAK). The GST-51-308 paxillin fusion protein supported binding of vinculin and metavinculin (116 and 150 kDa, respectively), pp125 Fak and its GST-51-308 paxillin fusion protein spanning residues 51-308 (GST-51-308) was incubated with a lysate of embryonic day 15 gizzard. The fusion protein and associated proteins were collected by binding to glutathione-agarose beads (lanes 3). Similar incubations were performed with GST (29 kDa) alone (lanes 2). These samples were co-electrophoresed with a sample of total embryonic gizzard lysate (lanes 1) and either stained with Coomassie Blue (CB) or blotted with antibodies to vinculin (VN), talin (TN) or pp125 Fak (FAK). The GST-51-308 paxillin fusion protein supported binding of vinculin and metavinculin (116 and 150 kDa, respectively), pp125 Fak and its truncated homologue p41Fak (125 and 41 kDa, respectively) but not talin (215 kDa). No binding was detected of these proteins to GST alone. The 75 kDa protein stained with Coomassie Blue in CB, lane 3 is derived from the bacteria. Composite of two blots. M, molecular mass markers (×10^3)).

![Fig. 7. Schematic representation of the paxillin domains. The position of each of the five potential SH2-binding domains is marked with a Y. The hatched box marks the putative SH3-binding domain. For clarity, additional tyrosine kinase and protein kinase C phosphorylation consensus sites have not been included. Note, the position of the amino terminus has not yet been determined.](attachment:image)
Cytoskeleton-associated signaling complexes. Perturbation of tyrosine phosphorylation/dephosphorylation may be critical pathways important in cytoskeletal organization and growth such may represent a point of convergence for signaling provides evidence that this molecule is capable of associating fragment of paxillin.

Presence of a binding site for vinculin within this 28 kDa protein, described in this report, is due to the association between these two proteins, as noted previously (Turner et al., 1990; Wood et al., 1992) and therefore it is likely that the interaction between pp125 Fak and the truncated non-catalytic form p41 Frnk , which is composed solely of the carboxyl terminus of pp125 Fak (Schaller et al., 1993), suggests that pp125 Fak may be targeted to focal adhesions through an association with paxillin. However, at present, we cannot exclude the possibility that the binding of pp125 Fak to the paxillin fusion protein is indirect. In contrast, a direct interaction between paxillin and vinculin has been documented previously (Turner et al., 1990; Wood et al., 1994) and therefore it is likely that the interaction between these two proteins, described in this report, is due to the presence of a binding site for vinculin within this 28 kDa fragment of paxillin.

In summary, the deduced amino acid sequence of paxillin provides evidence that this molecule is capable of associating with numerous structural and regulatory molecules, and as such may represent a point of convergence for signaling pathways important in cytoskeletal organization and growth control. Our identification of a physical association between paxillin and pp125 Fak provides further evidence that the selective, co-ordinate tyrosine phosphorylation of paxillin and pp125 Fak following integrin-mediated cell adhesion (Burrage et al., 1992), during embryonic development (Turner, 1991; Turner et al., 1993), and in response to certain growth factors (Zachary et al., 1992, 1993), is likely to be the result, at least in part, of direct phosphorylation of paxillin by pp125 Fak. The regulation of the interactions discussed above, through paxillin tyrosine phosphorylation/dephosphorylation, may be critical for the correct assembly close to the plasma membrane of cytoskeleton-associated signaling complexes. Perturbation of such interactions by SH2-containing oncoproteins like v-src or v-crk might be responsible for the disrupted cytoskeletal organization observed in transformed cells (Burrage, 1986) and, more significantly, the failure to process correctly extracellular signals important in controlling normal cell growth. Future work will be directed towards further characterization of paxillin’s multiple protein-binding domains and determining the precise role of paxillin in intracellular signaling mechanisms.

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REFERENCES


Guan, J.-L. and Shalloway, D. (1992). Regulation of focal adhesion-
associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 358, 690-692.


