Actopaxin, a New Focal Adhesion Protein That Binds Paxillin LD Motifs and Actin and Regulates Cell Adhesion

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Abstract. Paxillin is a focal adhesion adapter protein involved in the integration of growth factor– and adhesion-mediated signal transduction pathways. Paxillin LD motifs have been demonstrated to bind to several proteins associated with remodeling of the actin cytoskeleton including the focal adhesion kinase, vinculin, and a complex of proteins comprising p95PKL, PIX, and PAK (Turner, C.E., M.C. Brown, J.A. Perrotta, M.C. Riedy, S.N. Nikolopoulos, A.R. McDonald, S. Bagrodia, S. Thomas, and P.S. Leventhal. 1999. *J. Cell Biol.* 145:851–863). In this study, we report the cloning and initial characterization of a new paxillin LD motif–binding protein, actopaxin. Analysis of the deduced amino acid sequence of actopaxin reveals a 42-kD protein with two calponin homology domains and a paxillin-binding subdomain (PBS). Western blotting identifies actopaxin as a widely expressed protein. Actopaxin binds directly to both F-actin and paxillin LD1 and LD4 motifs. It exhibits robust focal adhesion localization in several cultured cell types but is not found along the length of the associated actin-rich stress fibers. Similar to paxillin, it is absent from actin-rich cell–cell adherens junctions. Also, actopaxin colocalizes with paxillin to rudimentary focal complexes at the leading edge of migrating cells. An actopaxin PBS mutant incapable of binding paxillin in vitro cannot target to focal adhesions when expressed in fibroblasts. In addition, ectopic expression of the PBS mutant and/or the COOH terminus of actopaxin in HeLa cells resulted in substantial reduction in adhesion to collagen. Together, these results suggest an important role for actopaxin in integrin-dependent remodeling of the actin cytoskeleton during cell motility and cell adhesion.

Key words: actin-binding protein • CH domains • focal adhesions • paxillin • hic-5

Introduction

Cell adhesion and migration are integral components of many biological processes including normal development and recovery from injury and infection. These processes are regulated in part by signals received by cells through the engagement of integrins with the extracellular matrix in combination with exposure to soluble growth factors (Clark and Brugge, 1995; Craig and Johnson, 1996). The integrin–extracellular matrix interaction catalyzes the recruitment of multiple cytoskeletal-associated proteins to the cytoplasmic face of structures called focal adhesions, triggering a physical linkage between the extracellular matrix and the actin-based cytoskeleton (Turner and Burridge, 1991; Jockusch et al., 1995). Some focal adhesion proteins such as vinculin, talin, and α-actinin function solely to provide a structural link between integrins and F-actin, whereas others such as the focal adhesion kinase (FAK),[1] Cas, and paxillin function as scaffold proteins and are involved in coordinating integrin-mediated signal transduction (Burridge and Chrzanowska-Wodnicka, 1996) associated with the regulation of cell motility, gene expression, and cell proliferation (Giancotti and Ruoslahti, 1999).

Paxillin is a multidomain adapter protein composed of protein–protein interaction motifs (Turner et al., 1990; Turner, 1998). The COOH terminus of paxillin is comprised of four LIM domains that mediate paxillin targeting to focal adhesions (Brown et al., 1996, 1998b) as well as binding to the tyrosine phosphatase PTP-PEST (Shen et al., 1998; Coté et al., 1999). The NH_2 terminus of paxillin supports the binding of vinculin and several protein tyrosine kinases including FAK, PYK2, Csk, and Src (Sabe et al., 1994; Turner and Miller, 1994; Schaller and Parsons,

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[1]*Abbreviations used in this paper:* aa, amino acid(s); Arf, ADP-ribosylation factor; CH, calponin homology; FAK, focal adhesion kinase; GST, glutathione *S*-transferase; HISM, human intestinal smooth muscle; PBS, paxillin-binding subdomain; RT, reverse transcriptase.

1995; Tachibana et al., 1995; Salgia et al., 1996). Paxillin also binds to α4 integrins (Liu et al., 1999).

We have identified five leucine-rich sequences (LDXLLXXL) within the NH_2 terminus of paxillin named LD motifs that are highly conserved between species and between other paxillin family members such as hic-5 and *Dictyostelium discoideum* PaxB (Brown et al., 1996, 1998a). The paxillin LD motifs serve as binding sites for both FAK and vinculin (Brown et al., 1996; Turner, 1998; Turner et al., 1999), and the E6 oncoprotein from papillomavirus (Tong et al., 1997; Vande Pol et al., 1998). Recently, we described a novel scaffolding function for the paxillin LD4 motif in the binding of a complex of proteins containing the ADP-ribosylation factor (Arf) GTPase–activating protein, paxillin-kinase linker (p95PKL), the p21-Cdc42/Rac GTPase–activated serine threonine kinase (PAK), Nck, and the guanine nucleotide exchange factor PIX/COOL (Turner et al., 1999). The p21 GTPases of the Rho family (Cdc42, Rac, and Rho) and their effectors such as PAK and ROK play a pivotal role in regulating remodeling of the actin cytoskeleton and change in gene expression in response to stimulation of quiescent cells with growth factors or after cell adhesion (Clark et al., 1998; Hall, 1998; Price et al., 1998; Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). More recently, the Arf family of small GTPases have also been implicated in remodeling of the actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Frank et al., 1998; Song et al., 1998) in addition to their role in vesicle transport (Donaldson and Klausner, 1994; Radhakrishna and Donaldson, 1997). Additional members of the Arf GAP family that also bind to paxillin have been reported recently including P95-APP1 (Di Cesare et al., 2000), GIT-1 (Premont et al., 2000), and PAG3 (Kondo et al., 2000). Thus, paxillin, through its multiple protein–protein interactions, likely serves as an important mediator of cytoskeletal reorganization and as a potential site of integration of Rho GTPase and Arf GTPase signaling (Norman et al., 1998; Turner et al., 1999).

Here we report the identification and characterization of another paxillin LD motif–binding protein, p42 actopaxin. We demonstrate that actopaxin, a tandem calponin homology (CH) domain–containing protein, binds directly to paxillin and F-actin and colocalizes with paxillin at focal adhesions and at the leading edge of migrating fibroblasts. Moreover, ectopic expression of either an actopaxin construct–containing mutation of the paxillin-binding subdomain (PBS) or the COOH-terminal half of actopaxin resulted in substantial reduction in the spreading/adhesion efficiency of HeLa cells on collagen. These data suggest an important role for actopaxin and/or the actopaxin–paxillin interaction either in the regulation of integrin's association with the extracellular matrix or in the initial organization of the actin cytoskeleton during cell adhesion and spreading.

Materials and Methods

Reagents and Antibodies

Rat collagen type I and α-actinin antibody were obtained from Sigma-Aldrich. Paxillin (clone 349) and $p130^{Cas}$ (clone 21) antibodies were obtained from Transduction Laboratories, paxillin Y118 antibody from Biosource International, actin antibody (clone C4) from Roche, and Xpress antibody from Invitrogen. GFP antibody was a generous gift of Dr. P. Silver (Dana-Farber Cancer Institute, Boston, MA).

Cell Culture and Transfection

Human intestinal smooth muscle (HISM) cells, rat embryo fibroblasts (REF-52), NIH3T3, HeLa, and Cos-7 cells were maintained in DME (Mediatech) supplemented with 10% (vol/vol) FBS (Atlanta Biologicals), 1 mM glutamine, and 50 U/ml penicillin/50 μg/ml streptomycin (Sigma-Aldrich) in a humidified chamber with 5% CO_2. CHO-K1 cells were cultured in modified Ham's F-12 (Mediatech) supplemented with 10% (vol/vol) FBS and 1% penicillin/streptomycin at 37°C. Rat intestinal epithelial cells (IEC-18) were maintained in DME supplemented with 5% (vol/vol) FBS, 4 mM L-glutamine, 0.1 U/ml insulin, and 50 U/ml penicillin/50 μg/ml streptomycin. Lipofectamine (GIBCO BRL)-mediated transfection of CHO-K1, HeLa, and Cos-7 cells was as described elsewhere (Brown et al., 1996).

Microsequencing, Cloning, and Mutagenesis of Actopaxin

Paxillin glutathione *S*-transferase (GST)-LD1 binding proteins from chicken smooth muscle tissue lysate were separated by SDS-PAGE and transferred to PVDF membrane as described previously (Turner et al., 1999). Protein bands, including a 42-kD band, were visualized by Ponceau S staining and excised. Proteolytic digestion, HPLC fractionation, and internal peptide sequencing were performed by Dr. John Leszyk (Worcester Foundation for Biomedical Research, Worcester, MA). Peptide sequencing revealed peptides with identical sequences with mouse and human EST clones in GenBank. The equivalent ESTs were obtained from Genome Systems and sequenced on both strands (BioResource Center, Cornell University, Ithaca, NY). Sequencing and alignment of the EST clones revealed a 1.4-kb sequence that contained all the peptide sequences derived from the microsequencing of the 42-kD band. The full coding sequences for actopaxin (amino acids [aa] 1–372, both mouse and human) were isolated by reverse transcriptase (RT)-PCR from NIH3T3 and HISM cells, using forward oligonucleotide primer 5′-CGTCTAGAATGGCCACATCCCCAC-3′ and reverse oligonucleotide primer 5′-CGTCTAGATCACTCCACATTCCGG-3′. A nucleotide tail coding for the XbaI restriction site (underlined nucleotides) was engineered at the 5′ end of each primer to facilitate in-frame cloning into the XbaI site of pEGFP-C1 (CLONTECH Laboratories, Inc.) or pcDNA3.1HisC (Invitrogen) vectors. The NH_2-terminal half (aa 1–222) and the COOH-terminal half of actopaxin (aa 223–372) were also cloned in-frame with the Xpress tag in the EcoRI site of pcDNA3.1HisC vector. A double point mutant of the PBS domain of actopaxin ($val^{282}gly/leu^{285}arg$) was generated using the Quick Change Mutagenesis kit (Stratagene) according to the instructions of the manufacturer. All actopaxin constructs were sequenced on both strands (BioResource Center, Cornell University, Ithaca, NY). The sequence data for human and mouse actopaxin are available from GenBank/EMBL/DDBJ under accession nos. AF264765 and AF264766, respectively.

Preparation of Fusion Proteins and Binding Assays

Individual GST fusion proteins of paxillin LD motifs, as well as GST wild-type paxillin (aa 1–559), were expressed in *Escherichia coli* (DH5α) and purified on glutathione–agarose beads as described previously (Turner et al., 1999). GST-actopaxin fusion proteins were generated by subcloning the full-length actopaxin (aa 1–372) into the EcoRI site of pGEX-1 vector (Amersham Pharmacia Biotech), the NH_2-terminal half (aa 1–222) into the BamHI sites of pGEX-2T, and the COOH-terminal half (aa 223–372) between the BamHI and EcoRI sites of pGEX-3X. Actopaxin fusion proteins were expressed in BL21pLysS cells (Novagen).

For all binding experiments, cells were lysed in lysis/binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1% NP-40, 10% glycerol) containing protease inhibitors (Roche), followed by clarification for 15 min at 15,000 *g*. Lysates (0.5–1 mg) were incubated for 4 h at 4°C with the GST-actopaxin or GST-paxillin LD proteins (50 μg) bound to glutathione–Sepharose 4B beads or with the appropriate antibodies coupled to protein A/G beads (Santa Cruz Biotechnology, Inc.). After incubation, the beads were washed three times with the same buffer, and bound proteins were separated by 12% SDS-PAGE and analyzed by Western blotting. In similar experiments, GST-paxillin, GST-paxillin LD motifs, or GST-actopaxin fusion proteins were incubated with either ^{35}S-metabolically labeled actopaxin or actopaxin PBS mutant generated by coupled in vitro transcription/translation (TNT; Promega). Binding proteins were analyzed on SDS-PAGE gels followed by enhanced fluorography using Amplify (Amersham Pharmacia Biotech).

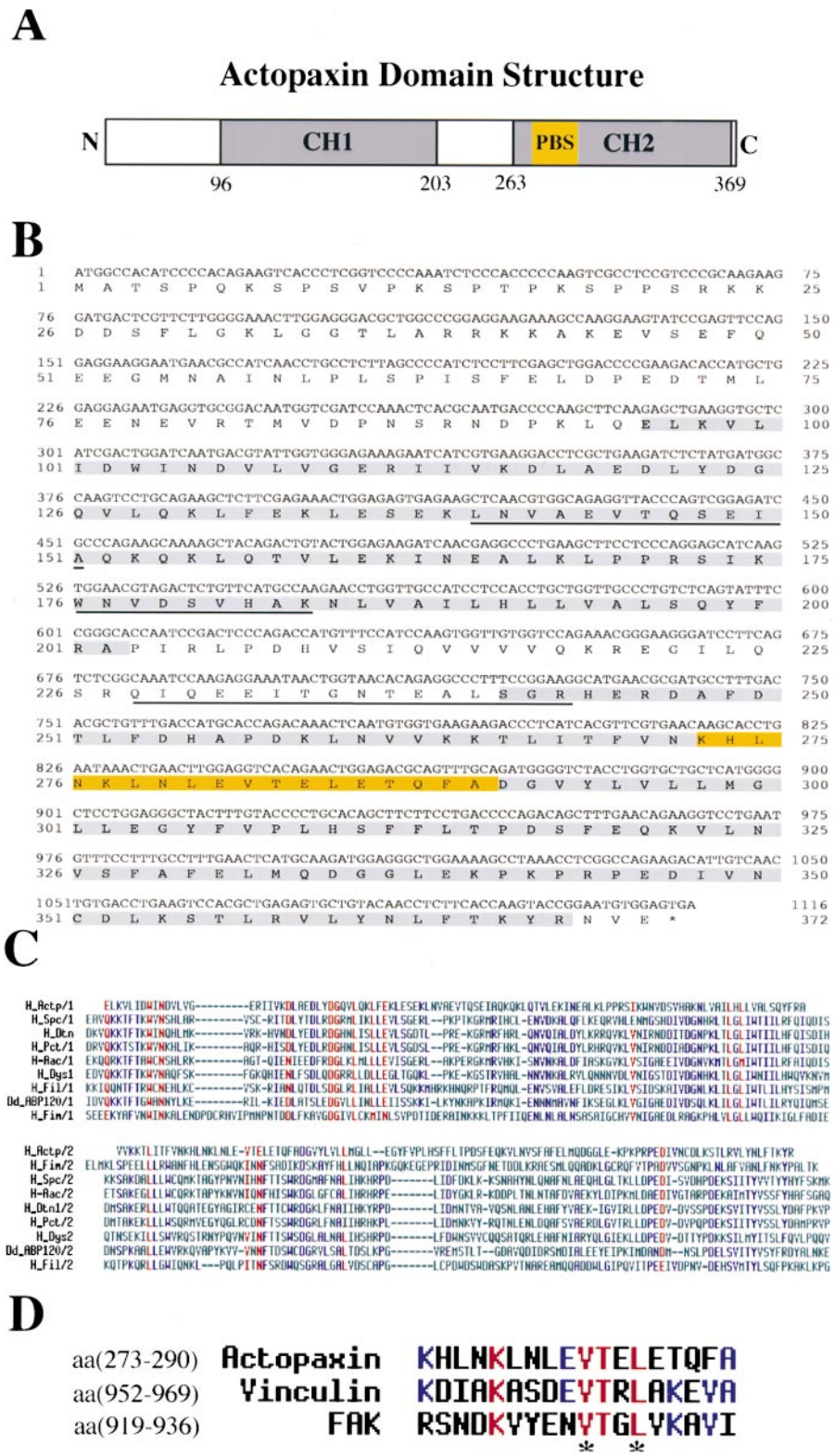

Figure 1. Sequence analysis of actopaxin. (A) Schematic representation of the domain structure of actopaxin showing the relative positions of CH domains (CH1 and CH2) and PBS sequence (PBS). (B) Nucleotide and deduced aa sequence of human actopaxin cDNA. The gray boxes highlight the CH domains. The aa sequences of the three peptides derived from the microsequencing of the 42-kD band are underlined. The PBS is highlighted in yellow. The sequence data are available from GenBank/EMBL/DDBJ databases under accession no. AF264765. (C) Sequence alignment of CH domains from different actin-binding proteins. The CH1 domains are aligned on the top and the CH2 domains on the bottom. Residues conserved in >90% of the sequences are colored red; residues conserved in >50% of the sequences are colored blue. The names, database accession numbers (SWISSPROT [SW] or SPTREMBL [SE]), and sequence residues are as follows: H_Actp, human actopaxin aa 96–202 and aa 262–368; H_Spc, human β spectrin, SW Q01082, aa 53–163 and aa 172–281; H-Aac, human α-actinin1 SW P12814, aa 30–140 and aa 143–253; H_Dtn_l, human dystonin isoform 1, SE Q13266, aa 34–143 and aa 150–258; H_Pct, human plectin, SE Q15149, aa 178–287 and aa 294–403; H_Dys, human dystrophin, SW P11532, aa 14–124 and aa 133–243; H_Fil, human nonmuscle filamin (ABP-280), SW P21333, aa 42–154 and aa 165–272; Dd_ABP-120, *D. discoideum* ABP-120, SW P13466, aa 11–122 and aa 124–230; H_Fim, human fimbrin, SW P13797, aa 119–241 and aa 260–379. The figure was produced with MULTALIN software (Corpet, 1988). (D) Sequence alignment of the PBS domains of actopaxin, vinculin, and FAK. Identical residues are colored red; residues conserved in at least two of the sequences are colored blue. Asterisks indicate the aa that have been reported previously to be critical for FAK binding to paxillin and which are mutated in the actopaxin PBS mutant.

Actin Cosedimentation Assays

Actin and α-actinin were obtained from Cytoskeleton. GST-actopaxin fusion proteins were prepared as described above and eluted from the glutathione-agarose beads with GST elution buffer (20 mM glutathione, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% β-mercaptoethanol). Before performing actin binding studies, all samples were dialyzed for 16 h at 4°C against 2,000 volumes of actin-binding buffer (10 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM DTT), followed by centrifugation in a Beckman Coulter airfuge (150,000 *g*) for 1 h at room temperature to eliminate protein aggregates. F-actin was prepared by polymerization of G-actin in the presence of binding buffer for 1 h at room temperature. F-actin (23 μM) was incubated with GST-actopaxin fusion protein (1 μM), purified α-actinin (2 μM), BSA (2 μM), or GST (50 μM) for 30 min at room temperature. After incubation, the samples were centrifuged in a Beckman airfuge (150,000 *g*) for 1 h at room

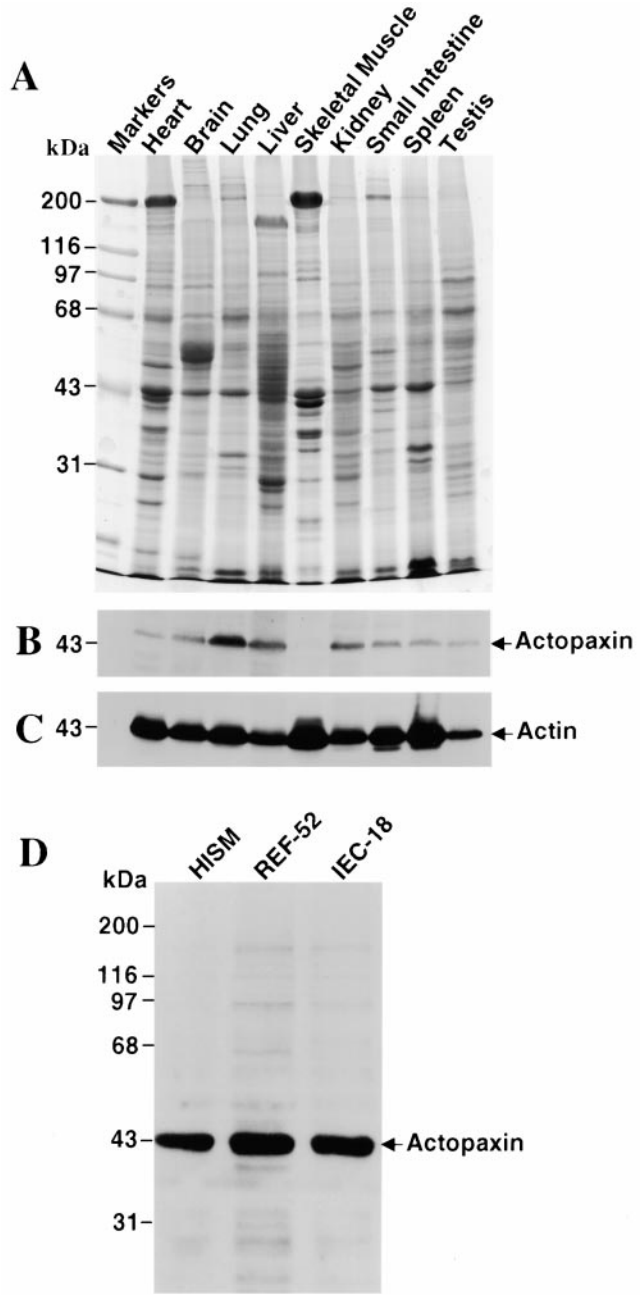

Figure 2. Expression of actopaxin protein in various tissues and cell lines. Organs were dissected from a healthy adult rat, homogenized, and lysed in SDS-PAGE sample buffer. 50 μg of each lysate was loaded per lane. (A) Coomassie blue staining of the gel. (B) Western blotting of an identical gel shown in A probed with the actopaxin antibody. (C) The same blot was stripped and reprobed with an actin antibody to indicate relative loading among lanes. (D) Western blot of total lysates (50 μg) from HISM cells, rat embryo fibroblasts (REF-52), and rat intestinal epithelial cells (IEC-18) probed with the actopaxin antibody.

temperature to pellet the actin filaments and associated proteins. The supernatant and pellet fractions were analyzed by 10% SDS-PAGE and visualized with Coomassie blue.

RNA Isolation, PCR, RT-PCR, and Northern Blot Analysis

Total RNA from HISM and NIH3T3 cells was prepared using the Trizol reagent (GIBCO BRL) according to the manufacturer's protocol. cDNAs were prepared from total RNAs using SuperScriptII (GIBCO BRL). PCR and RT-PCR were performed using the Expand High Fidelity PCR system (Roche). A human tissue Northern blot containing 2 μg of poly(A)$^+$ RNA (CLONTECH Laboratories, Inc.) was probed with a ^{32}P-labeled actopaxin probe encompassing the actopaxin ORF and hybridized in QuickHyb hybridization solution according to the manufacturer's instructions (CLONTECH Laboratories, Inc.).

Antibody Preparation

GST-actopaxin was purified on glutathione–Sepharose 4B (Amersham Pharmacia Biotech), eluted from the beads, and dialyzed against phosphate-buffered saline. Rabbits were immunized by repeated subcutaneous injections of the purified fusion protein (300 μg) suspended in Freund's Adjuvant.

Immunofluorescence Microscopy

Indirect immunofluorescence analysis was performed as described previously (Brown et al., 1996). Photographs were taken on a ZEISS Axiophot photomicroscope equipped with epifluorescence illumination using Tmax 400 film (Eastman Kodak Co.). Images were scanned using Coolscan II (Eastman Kodak Co.) and processed using Adobe Photoshop® v3.0.5.

Fibroblast Wound-healing Assay

To stimulate the formation of lamellipodia, scrape-wound healing assays were performed on HISM cells grown on coverslips. Confluent, serum-starved monolayers of HISM cells were wounded by scraping away a swath of cells with a P1000 pipet tip. The coverslips were rinsed twice with phosphate-buffered saline to remove wound-derived cell aggregates, and then incubated in complete media for 3 h at 37°C, 5% CO_2. The coverslips were subsequently fixed and processed for immunofluorescence microscopy as described previously (Brown et al., 1996).

Adhesion and Spreading Assays

HeLa cells, transiently transfected with Xpress-tagged actopaxin constructs or with an Xpress-tagged β-galactosidase plasmid (Invitrogen), were resuspended 18 h after transfection, washed once in complete medium with the addition of 10 μg trypsin inhibitor (Sigma-Aldrich), and then washed three times with serum-free medium. Thereafter, the cells were resuspended in serum-free medium containing 1% BSA and rocked for 1 h at 37°C. The cells were then plated on collagen type I (10 μg/ml) for 45, 90, or 180 min in serum-free medium, or for 12 h in complete medium. For each time point, slips were fixed in 3.7% formaldehyde and processed for immunofluorescence with Xpress antibody. To monitor transfection efficiency before the spreading assay, a coverslip (present in transfection plates) was processed for immunofluorescence before trypsinization. Adhesion and spreading efficiency was expressed as the percentage of adherent transfected cells at each time in relation to the number of adherent transfected cells after 12 h in complete medium (designated 100%). A minimum of 200 cells were analyzed per construct per time point. The results represent the mean of three experiments ± SD.

Results

Molecular Cloning of Actopaxin

Recently, we have described the identification and characterization of several paxillin LD4 motif–binding proteins (Turner et al., 1999). During the course of these studies additional LD motif–binding proteins were detected. Here, we describe the cloning and characterization of one of these proteins, a 42-kD protein initially identified through its association with the paxillin LD1 motif. Three peptide sequences obtained from microsequencing of the 42-kD LD1–binding protein (underlined, Fig. 1 B) indicated 100% identity with hypothetical translation products of mouse ESTs (sequence data available from GenBank/EMBL/DDBJ under accession nos. AA013795 and AA073392). These ESTs were obtained and sequenced in their entirety and were found to have overlapping sequence information. The predicted ORF

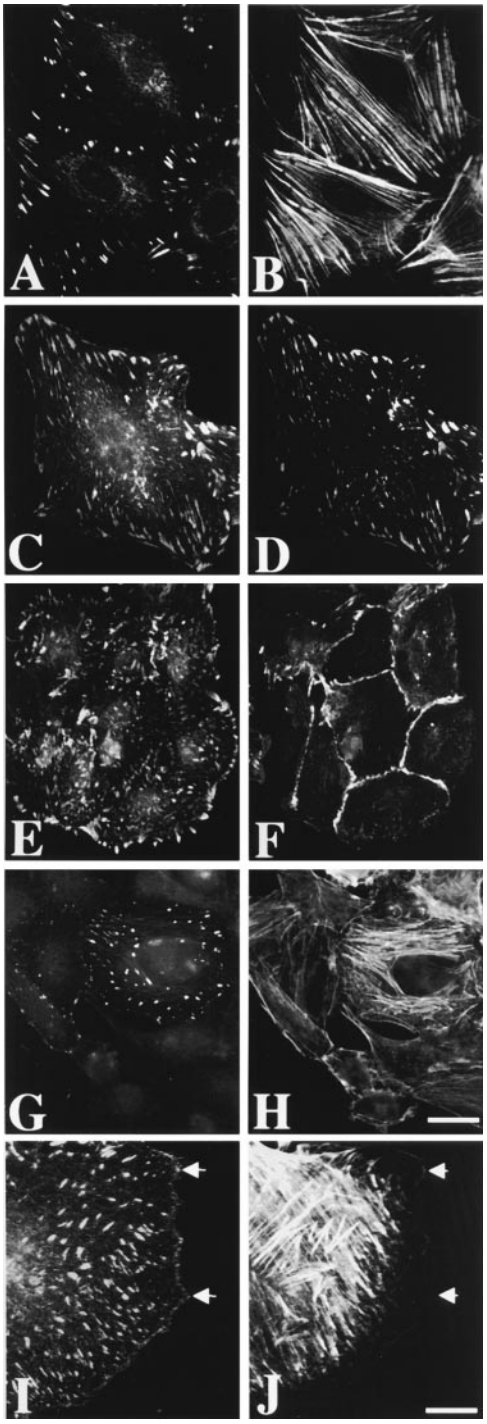

Figure 3. Subcellular distribution of actopaxin. Immunofluorescence staining with actopaxin antibody of (A) rat embryo fibroblasts (REF-52), (C) HISM cells, and (E) rat intestinal epithelial cells (IEC-18). Cells were stained simultaneously with rhodamine phalloidin to visualize actin stress fibers (B), paxillin antibody to demonstrate colocalization of actopaxin with paxillin at focal adhesions (D), and β-catenin antibody to demonstrate that actopaxin is absent from cell–cell contacts (F). Xpress-tagged actopaxin was transfected in CHO-K1 cells (G and H). The cells were stained simultaneously with anti-Xpress antibody to detect the transfected actopaxin (G) and rhodamine phalloidin (H) to visualize actin stress fibers. Xpress-actopaxin was enriched at focal adhesions at the ends of actin stress fibers (G and H). Actopaxin is recruited to the focal complexes (arrows) at the leading edge of migrating fibroblasts (I and J). HISM cells were stimulated to migrate by scrape wounding a confluent monolayer of cells. 3 h after wounding, the cells were stained for immunofluorescence microscopy with actopaxin antibody (I) and rhodamine phalloidin (J) to visualize actin stress fibers. Bars: (A–H) 5 μm; (I and J) 2 μm.

of the combined EST sequences encodes a protein of 372 aa with an estimated molecular mass of 42.1 kD. Both the human and mouse clones (sequence data available from GenBank/EMBL/DDBJ under accession no. AF264765/6) containing the entire ORF for this protein were cloned by RT-PCR from HISM cells or mouse NIH3T3 fibroblast cells, respectively. We have named this 42-kD protein actopaxin in recognition of its actin- and paxillin-binding properties as demonstrated below.

Protein Sequence and Structural Features of Actopaxin

Comparison of the deduced aa sequences of human and mouse actopaxin indicates that this novel protein is highly conserved between species, sharing 98.9% identity at the aa level. Computer-assisted databank searches revealed that actopaxin shares highest homology with two recently reported sequences: AK001655, 98% identity; and AL159142, 75% identity. In addition, actopaxin shares relatively high homology (48% identity) with a *Caenorhabditis elegans* protein, which is related to α-actinin (AF016687), and 27% identity with most of the α-actinin family members. A closer examination of the domain structure of actopaxin revealed the presence of two CH domains in tandem array designated CH1 (aa 96–203) and CH2 (aa 263–369) (Fig. 1, A and B). CH domains, especially when presented in tandem, have been shown to function as F-actin–binding domains in several structural proteins including α-actinin, spectrin, and fimbrin (Banuelos et al., 1998). An alignment of the CH domains of actopaxin with those of representative members of the CH domain family demonstrate significant conservation of consensus forming residues (Fig. 1 C).

Consistent with a model in which paxillin LD motifs interact with their binding partners through conserved sequences known as PBS (Wood et al., 1994; Tachibana et al., 1995; Brown et al., 1998a; Turner et al., 1999), we have identified a PBS within the CH2 domain of actopaxin (highlighted in green, Fig. 1, A and B). The actopaxin PBS (aa 273–290) displays 38.8% identity (55.5% conservation) with the vinculin PBS (aa 951–989) and 27.7% identity (33.3% conservation) with the FAK PBS. Sequence alignment of the actopaxin PBS sequence with the PBS sequences of vinculin and FAK revealed conservation among all three proteins. Valine^{928} and leucine^{931} of FAK have previously been shown to be essential for binding to paxillin (Tachibana et al., 1995) (Fig. 1 D). The corresponding aa of actopaxin, valine^{282}, and leucine^{285} were mutated to glycine and arginine, respectively, and used in experiments described in Figs. 8 and 9.

Structural predictions provided by the COILS algorithm (Lupas et al., 1991) define actopaxin as a protein with a theoretical pI of 5.49 and a predominantly coiled-coil α helical structure, displaying an aa composition with a high content of leucine (13.4%), glutamic acid (8.9%), and lysine (8.6%). There is also a high proline

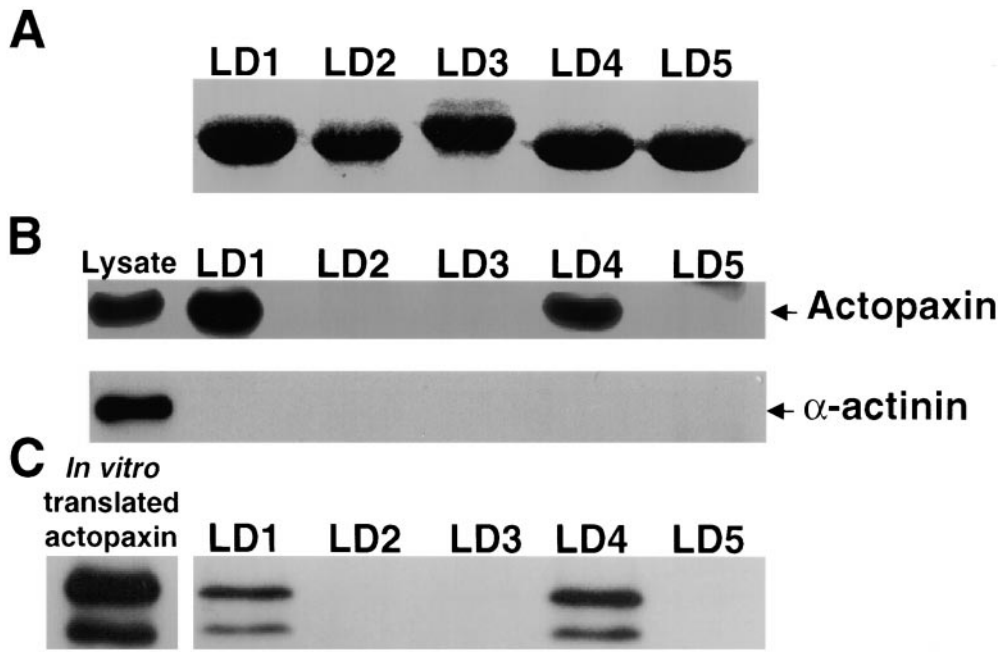

Figure 4. Actopaxin binds to the LD1 and LD4 motifs of paxillin. (A) Coomassie blue staining of the GST-paxillin LD motif fusion proteins. (B) Each paxillin LD motif fusion protein was incubated with 200 μg total lysate from REF-52 cells. Coprecipitating proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with actopaxin antibody (top). The same blot was stripped and probed with α-actinin antibody (bottom). (C) Individual GST-LD domains of paxillin were incubated with in vitro–translated ^{35}S-labeled actopaxin and binding proteins, precipitated using glutathione-agarose beads, resolved by SDS-PAGE, and visualized by fluorography.

content at its extreme NH_2 terminus (aa 1–95). Motif analysis of actopaxin indicates multiple potential phosphorylation sites for the cell cycle regulatory kinase $p34^{cdc2}$ in this NH_2 terminus region (aa 1–95), a potential tyrosine phosphorylation site (aa 117–124), and multiple consensus sequences for protein kinase C and casein kinase II phosphorylation.

Messenger RNA and Protein Expression of Actopaxin

The expression pattern of actopaxin mRNA in various human adult tissues was examined by Northern blot analysis at high stringency using the human actopaxin ORF as a probe. Although ubiquitous, actopaxin mRNA was primarily expressed in the heart and pancreas. Several transcripts ranging from 1.4 kb to ~8 kb were detected (data not shown). The presence of these transcripts suggests that actopaxin is a member of a larger family of proteins.

To examine the expression pattern of actopaxin protein, a polyclonal antiserum was produced against a GST fusion protein of full-length actopaxin (aa 1–372). Western blotting of total cell lysates prepared from several rat tissues and cell lines indicated the presence of a major protein migrating just above the 42-kD actin band in all tissues examined (Fig. 2, A–C) and in all cell lines tested (Fig. 2 D). The absence of actopaxin from the skeletal muscle sample is most likely due to the absence in this particular preparation of myotendinous junctions, a specialized region at the end of skeletal muscle fibers enriched in focal adhesion proteins (Tidball et al., 1986; Turner et al., 1991).

Actopaxin Subcellular Localization

To evaluate the subcellular distribution of actopaxin, REF-52, HISM, and IEC-18 cells were processed for immunofluorescence microscopy using the actopaxin antibody. In all cell lines examined, actopaxin demonstrated robust focal adhesion staining (Fig. 3, A, C, and E) where it colocalized with paxillin (Fig. 3, C and D). Interestingly, although actopaxin also binds F-actin (see below), no significant actopaxin was detected along the length of the actin-rich stress fibers (i.e., outside the confines of the focal adhesions). Similar to paxillin, actopaxin did not localize to β catenin–containing cell–cell adherens junctions of epithelial cells (IEC-18) (Fig. 3, E and F). A variable amount of perinuclear staining for actopaxin was observed in all cell types examined.

To confirm the subcellular distribution of actopaxin, a cDNA of actopaxin linked to the NH_2-terminal Xpress epitope of the pcDNA3HisC vector was transfected into CHO-K1 cells. Xpress-tagged actopaxin localized to focal adhesions at the ends of actin stress fibers (Fig. 3, G and H). The same results were obtained with a GFP–actopaxin construct (data not shown).

In addition to its localization to focal adhesions, paxillin has been demonstrated to localize to the small integrin-based adhesions called focal complexes found at the ventral surface of newly formed lamellipodia (Nobes and Hall, 1995). To determine if actopaxin is present in these highly dynamic structures, a wound-healing assay was performed on confluent monolayers of HISM cells. Cells were scraped from a region of the glass coverslip and the remaining cells were allowed to migrate into the wound. Staining of the migrating cells with the actopaxin antibody demonstrated that as with paxillin, actopaxin is distributed in discrete focal complexes at the extreme leading edge of the cells at the base of the lamellipodia, as well as in more robust focal adhesions within the body of the cells (Fig. 3, I and J). These results potentially implicate actopaxin in the early events of cytoskeletal reorganization that accompany cell migration.

Actopaxin Selectively Binds Paxillin LD1 and LD4 Motifs

Our previous characterization of paxillin LD–binding proteins has revealed that although some binding proteins are

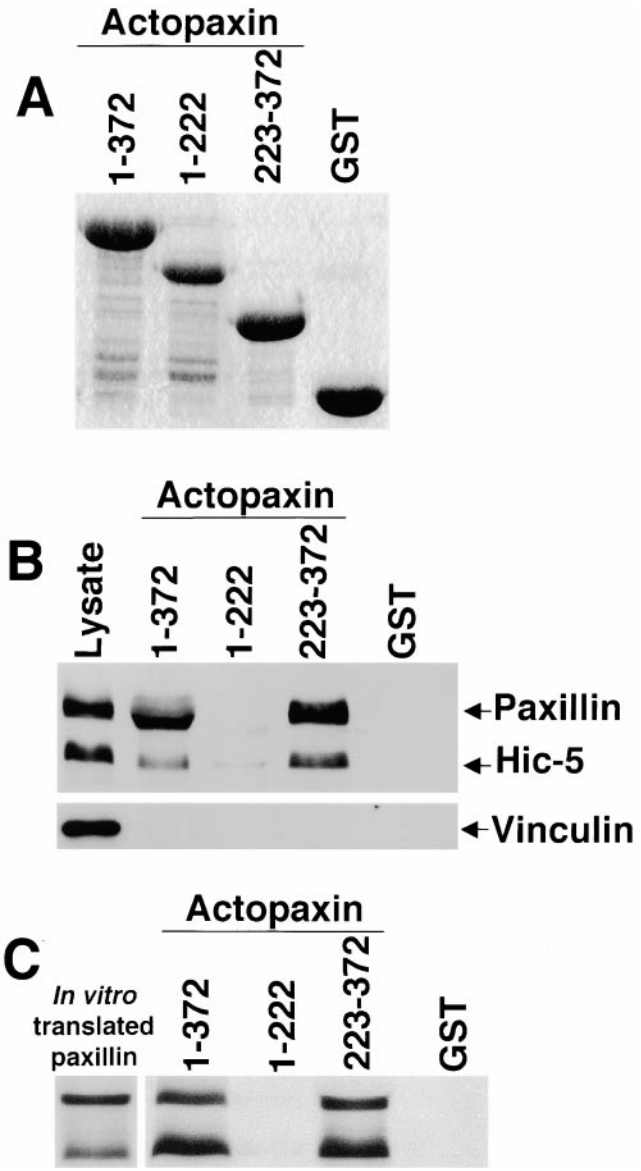

Figure 5. The second CH domain of actopaxin binds directly to paxillin and hic-5 in vitro. (A) Coomassie blue–stained gel of GST-actopaxin fusion proteins wild-type actopaxin (1–372), NH_2 terminus actopaxin (1–222), COOH terminus actopaxin (223–372). (B) GST-actopaxin fusion proteins shown in A were incubated with 300 μg of total lysate from REF-52 cells. Coprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with paxillin antibody (clone 349) that recognizes paxillin and hic-5 (top). The same blot was stripped and reprobed with vinculin antibody (bottom). (C) Paxillin was ^{35}S-metabolically labeled in vitro by coupled transcription/translation and tested for its ability to bind directly to GST-actopaxin fusion proteins.

restricted to a single LD motif (e.g., p95PKL to LD4), others bind to multiple LD motifs (vinculin to LD1, LD2, and LD4 and FAK to LD2 and LD4) (Turner et al., 1999). To determine if actopaxin binds exclusively to LD1, each paxillin LD motif was synthesized as a GST fusion protein (Fig. 4 A) and incubated with smooth muscle cell lysates in a precipitation binding experiment followed by Western blotting with actopaxin antibody. Actopaxin bound primarily to LD1 but also to LD4. Reprobing of the blot with α-actinin antibody revealed that α-actinin was unable to bind any paxillin LD motif (Fig. 4 B), thus confirming the specificity of actopaxin–paxillin binding. Alternatively, the same paxillin LD motif fusion proteins were incubated with in vitro–translated ^{35}S-labeled full-length actopaxin to demonstrate direct binding to paxillin LD1 and LD4 (Fig. 4 C).

Actopaxin Binds Paxillin and Hic-5 In Vitro

To delineate the paxillin-binding site on actopaxin, GST fusion proteins containing actopaxin wild-type (aa 1–372), actopaxin NH_2 terminus (aa 1–222), actopaxin COOH terminus (aa 223–372), and GST alone (Fig. 5 A) were incubated with REF-52 lysates and the precipitates were analyzed by Western blotting. The blots were probed with a paxillin antibody that recognizes both paxillin and the related 50-kD protein hic-5 (Brown et al., 1996; Hagmann et al., 1998). Both paxillin and hic-5 were detected in coprecipitates using full-length and COOH terminus actopaxin fusion proteins (Fig. 5 B). This robust binding of paxillin to the COOH terminus (aa 223–372) region of actopaxin is consistent with the presence of a PBS domain within that domain (see Fig. 1). To control for nonspecific binding, the blots were reprobed with vinculin antibody. No signal in any precipitation lane was obtained, thus excluding an actopaxin–vinculin association. To confirm these results and demonstrate a direct association between actopaxin and paxillin, GST-actopaxin fusion proteins were incubated with in vitro–translated ^{35}S-labeled full-length paxillin. Bound proteins were detected by SDS-PAGE followed by fluorography. Binding to paxillin occurred only with full-length actopaxin and the actopaxin COOH terminus fusion protein (Fig. 5 C).

Actopaxin is an F-Actin–binding Protein

The presence of tandem CH domains in several actin-binding proteins and the significant homology of actopaxin with spectrin and α-actinins suggest that actopaxin may associate directly with F-actin. To test this hypothesis, actin cosedimentation assays were performed using a GST fusion protein of wild-type actopaxin. α-Actinin was used as a positive control for actin binding, and GST and BSA controlled for nonspecific actin interactions. In the absence of F-actin, the GST-actopaxin fusion protein was found in the supernatant. In the presence of actin, however, a significant portion of the GST-actopaxin fusion protein was found to cosediment with F-actin (Fig. 6).

In Vivo Association of Actopaxin with Paxillin and Actin

To demonstrate an association in vivo between actopaxin, paxillin, and actin, GFP-actopaxin was transfected into Cos-7 cells. 48 h after transfection, total lysates from transfectants were immunoprecipitated with GFP antibody or with mouse IgG. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose, and the immunoblots were probed sequentially with paxillin/hic-5, GFP, $p130^{Cas}$, and actin antibodies. Paxillin and hic-5, as well as actin, coprecipitated with GFP-actopaxin but not

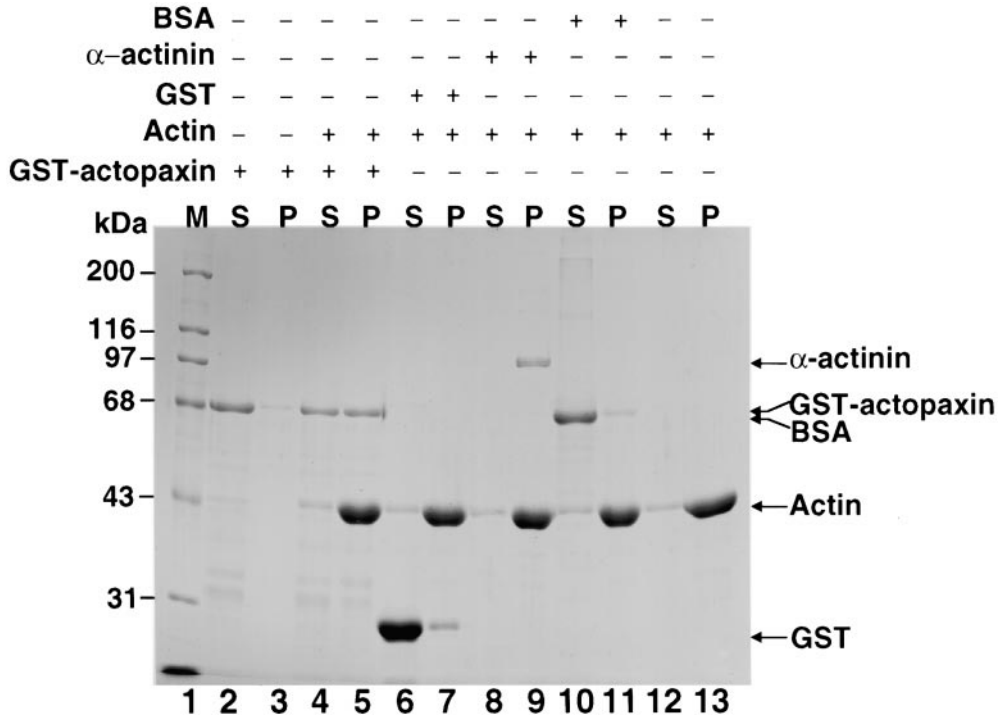

Figure 6. Actin-binding activity of GST-actopaxin. The ability of actopaxin to associate with F-actin in vitro was examined by actin cosedimentation assays using GST-actopaxin fusion protein. The following recombinant proteins were incubated with skeletal muscle F-actin: GST-actopaxin (lanes 4–5), GST (lanes 6–7), α-actinin (lanes 8–9), and BSA (lanes 10–11). GST-actopaxin (lanes 2–3) and F-actin (lanes 12–13) were also incubated in the absence of F-actin and GST-actopaxin, respectively. The supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE on 10% gel stained with Coomassie blue. Molecular weight markers (M) are shown (lane 1).

the control IgG. In contrast, $p130^{Cas}$ was not detected in the immunoprecipitates (Fig. 7 A).

To evaluate the interaction between endogenous actopaxin and paxillin, coimmunoprecipitation experiments were performed using REF-52 or HISM cells. Total lysates from both of these cell lines were incubated with actopaxin antibody or with control rabbit IgG. Immunoprecipitates were probed on Western blots with paxillin and α-actinin antibodies. Paxillin was efficiently coimmunoprecipitated with actopaxin antibody, whereas α-actinin failed to bind (Fig. 7 B).

Abrogation of the Actopaxin–Paxillin Association Renders Actopaxin Incapable of Localizing to Focal Adhesions

To investigate the importance of the actopaxin–paxillin association in the localization of actopaxin to focal adhesions, we generated a double point mutation within the PBS domain of actopaxin ($val^{282}gly/leu^{285}arg$). These two residues are conserved in the PBS domains of vinculin and FAK PBS (see Fig. 1 D) and, in the case of FAK, are essential for paxillin binding (Tachibana et al., 1995). To evaluate the ability of the actopaxin PBS mutant to bind paxillin, GST-paxillin fusion protein was incubated with in vitro–transcribed/translated ^{35}S-labeled actopaxin or the ^{35}S-labeled actopaxin PBS mutant. Bound proteins were detected by SDS-PAGE followed by fluorography. Binding of paxillin occurred only with wild-type full-length actopaxin and not with the actopaxin PBS mutant (Fig. 8 A).

When the actopaxin PBS mutant was transfected into either CHO-K1 or HeLa cells, it was unable to localize to focal adhesions, instead exhibiting a diffuse perinuclear and cytoplasmic distribution (Fig. 8 B). These results clearly demonstrate the importance of the actopaxin–paxillin association in the efficient recruitment of actopaxin to focal adhesions.

Actopaxin Is Involved in Cell Adhesion and Spreading of HeLa Cells

Cell adhesion and spreading are primarily mediated by integrin adhesion to the extracellular matrix and involve substantial reorganization of the actin cytoskeleton. The presence of actopaxin in focal adhesions and focal complexes, and its ability to link paxillin to actin, prompted us to investigate whether actopaxin is involved in the process of cell adhesion. For this purpose, Xpress-tagged wild-type (aa 1–372) actopaxin, the NH_2 terminus (aa 1–222), the COOH terminus (aa 223–372), the actopaxin PBS mutant, and β-galactosidase were transfected into HeLa cells and used in cell adhesion assays to collagen. The full-length (aa 1–372) construct was efficiently localized to focal adhesions within 45 min of postplating (Fig. 9 A, a). In contrast, the NH_2-terminal half of actopaxin (aa 1–222) showed a diffuse pattern of cytoplasmic localization at either 45 min or 12 h postplating (Fig. 9 A, b and j). Interestingly, the COOH-terminal half of actopaxin that contains the functional PBS domain (Figs. 5 and 8) localized weakly to focal adhesions after 12 h postplating (Fig. 9 A, k), confirming the presence of focal adhesion binding activity within this region of the protein. The actopaxin PBS mutant also showed a diffuse cytoplasmic staining without any evidence of focal adhesion staining at all time points postplating (Fig. 9 A, d and l). Visualization of the actin cytoskeleton in the transfected cells with rhodamine phalloidin indicated that overexpression of the COOH-terminal half of actopaxin or the actopaxin PBS mutant results in a partial reduction in actin filament content during the initial phases of cell spreading (Fig. 9 A, g and h). No significant differences were observed in the actin cytoskeleton with any of the transfectants 12 h postplating (Fig. 9 A, m–p). In contrast, the localization of paxillin to focal adhesions was unaffected by ectopic expression of any actopaxin construct (Fig. 10 and data not shown).

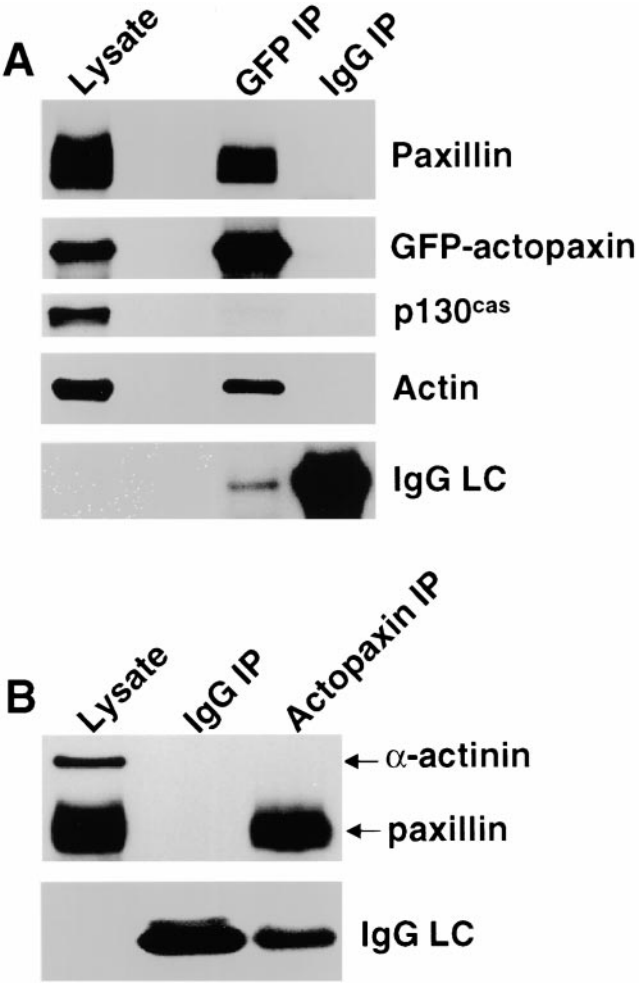

Figure 7. Actopaxin binds to paxillin and actin in vivo. (A) GFP-actopaxin was transfected into Cos-7 cells. After lysis, proteins were immunoprecipitated from 400 μg of total lysate with an antibody to either GFP or IgG control. 20 μg of total lysate and the immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blotting with antibodies to paxillin, GFP, actin, and $p130^{Cas}$. (B) Coimmunoprecipitation of paxillin with endogenous actopaxin. Asynchronously growing adherent HISM cells were lysed in coimmunoprecipitation buffer. Actopaxin and IgG immunoprecipitations were performed from 500 μg lysates and subjected to SDS-PAGE followed by Western blotting with antibodies to paxillin and α-actinin. Paxillin was coimmunoprecipitated with the actopaxin antibody in contrast to the control IgG. No binding of α-actinin to either actopaxin or IgG coimmunoprecipitates was observed. IgG LC, immunoglobulin light chain.

Interestingly, overexpression of either the actopaxin COOH terminus or the PBS mutant resulted in a substantial reduction in cell adhesion and spreading efficiency at 45 min postplating compared with cells expressing wild-type full-length actopaxin, NH_2 terminus actopaxin, or β-galactosidase (see Fig. 9 C). Transfectants of each of the actopaxin constructs or β-galactosidase were also assayed after 90 or 180 min postplating, and their adhesion efficiencies were analogous to the results at the 45-min time point (data not shown). Together, these data provide quantitative evidence that actopaxin plays an important role in the adhesion and spreading process.

Discussion

Previously, we identified five leucine-rich repeating sequences within the NH_2 terminus of paxillin, termed LD motifs (Brown et al., 1996, 1998a). Consistent with our proposed role for paxillin as a molecular adapter protein involved in integrin-mediated cytoskeletal organization and intracellular signaling, we determined that individual LD motifs could serve as independent and selective binding interfaces for other focal adhesion proteins including vinculin and FAK, as well as a novel Arf GAP protein p95PKL (Turner et al., 1999). In this study, we have identified and characterized a new paxillin LD1- and LD4-binding protein called p42 actopaxin. Actopaxin, which binds to paxillin through its PBS domain and colocalizes with paxillin to focal adhesions and focal complexes, was also found to bind directly to F-actin, suggesting a role for this protein in actin cytoskeleton and focal adhesion assembly/signaling associated with cell adhesion to the extracellular matrix. Indeed, overexpression of an actopaxin mutant defective in paxillin binding retarded the ability of cells to adhere and spread on the extracellular matrix.

Actopaxin is composed primarily of two CH domains. The CH domain, a module of ~110 aa named after the muscle regulatory protein calponin (Castresana and Saraste, 1995), is a common feature of actin-binding proteins (Banuelos et al., 1998). In general, two CH domains (designated CH1 and CH2) arranged in tandem constitute a functional actin-binding domain in several families of structural proteins including spectrin, α-actinin, filamin, fimbrin, and dystrophin (Stradal et al., 1998). These structural actin-binding proteins generally dimerize and thereby cross-link actin filaments into bundles or actin meshworks. Although actopaxin binds F-actin in in vitro cosedimentation assays, it is unclear at this time whether actopaxin undergoes a similar dimerization.

Actopaxin also contains a functional paxillin-binding PBS domain that contains several residues conserved within the PBS domains of vinculin and/or FAK (Tachibana et al., 1995; Brown et al., 1996). Comparison with the crystal structure analysis of filamin and spectrin CH domains (Carugo et al., 1997; Banuelos et al., 1998) suggests that the PBS domain of actopaxin, which lies between the first and second α-helix of the CH2 domain, is in a solvent-exposed hydrophilic loop and is thereby accessible for protein binding. Interestingly, this extended loop is present in only a limited number of CH domain–containing proteins (Banuelos et al., 1998), indicating that, although it is not required for the actin binding function of CH domains per se, it may indeed function as an additional protein-binding site in those proteins in which it is present. Furthermore, fimbrin, another actin-binding focal adhesion protein comprised almost totally of CH domains (four), has recently been shown to bind monomers of the intermediate filament protein vimentin via a region within its first CH domain (Correia et al., 1999), suggesting that CH domains likely serve as multiple protein-binding modules.

Finally, actopaxin contains multiple potential $p34^{cdc2}$ kinase phosphorylation sites within its NH_2 terminus. Phosphorylation of this domain of actopaxin may result in conformational changes within the protein, affecting its binding to actin and/or paxillin and thereby contributing to the disassembly of focal adhesions and stress fibers that

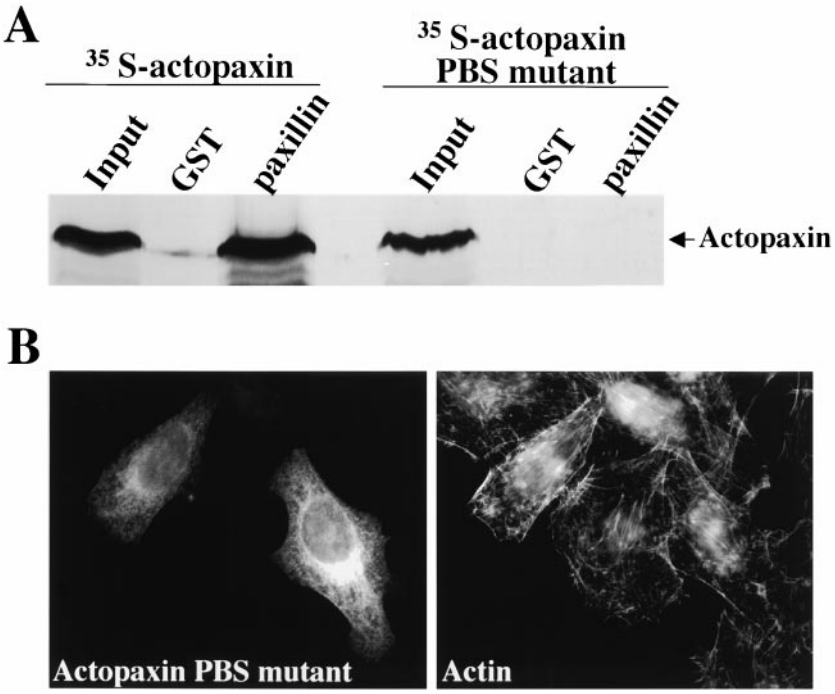

Figure 8. Actopaxin-PBS mutant does not bind paxillin in vitro and does not localize to focal adhesions. (A) GST or GST-paxillin was incubated with either in vitro–translated ^{35}S-labeled Xpress-actopaxin or ^{35}S-labeled Xpress-actopaxin PBS mutant, and binding proteins were resolved by SDS-PAGE and visualized by fluorography. (B) Xpress-actopaxin PBS mutant was transfected into HeLa, and the cells were stained simultaneously with anti-Xpress antibody to detect the transfected actopaxin and rhodamine phalloidin to visualize actin stress fibers. There was no evidence of focal adhesion localization for the actopaxin PBS mutant, although some perinuclear staining was detected. Bar, 5 μm.

occurs in adherent cells immediately before mitosis (Resnick et al., 1997; Kay et al., 2000).

The restricted localization of actopaxin to focal adhesions, rather than along the length of the associated actin-rich stress fibers, suggests that actopaxin may play an important role in linking these actin filaments to the plasma membrane–associated protein complex. Furthermore, actopaxin colocalizes with paxillin in the rudimentary focal complexes formed at initial cell contact sites with the extracellular matrix formed during lamellipodial extension at the leading edge of migrating cells (Nobes and Hall, 1995). The absence of well-organized actin filaments at focal complexes suggests that actopaxin, along with vinculin, another paxillin- and actin-binding protein, may contribute directly to the initial nucleation of actin assembly at these sites. Importantly, downregulation of proteins that perform this function, such as vinculin, can lead to a transformed phenotype (Rodriguez Fernandez et al., 1992), suggesting that this class of proteins plays a critical role in normal cell adhesion and growth control. Similarly, disruption of the actin cytoskeleton after infection of cells by the papillomavirus may be the result of binding of the viral oncoprotein E6 to paxillin LD motifs (Tong et al., 1997; Vande Pol et al., 1998) thereby causing the functional displacement of actopaxin and vinculin.

The identification of actopaxin extends the repertoire of paxillin-associated proteins involved in the modulation of the actin cytoskeleton. These paxillin LD motif–binding proteins fall into two major classes. The first includes actopaxin and vinculin, proteins that interact directly with actin and likely provide important structural links to the microfilament system. The second comprises important regulatory molecules associated with remodeling of the actin cytoskeleton by the Rho and Arf small GTPase families. For example, a complex of proteins binding to paxillin LD4 contains a putative activator of Rac/Cdc42, the nucleotide exchange factor, PIX/COOL (Bagrodia et al., 1998; Manser et al., 1998), and PAK, an effector of Rac and Cdc42 function associated with lamellipodia and filopodia extension (Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). These proteins are linked to paxillin through novel an Arf GAP protein, PKL (Turner et al., 1999), which is related to the Git family (Premont et al., 2000), APP-1 (Di Cesare et al., 2000), and PAG3 (Kondo et al., 2000). Although Arf GTPases are considered to function primarily in vesicle transport, they have recently been implicated in modifying the actin cytoskeleton through direct stimulation of lamellipodia formation (Song et al., 1998), potentially through coordinated activation of Rac (Di Cesare et al., 2000; Donaldson and Jackson, 2000) or by regulating the translocation to the plasma membrane of focal adhesion proteins such as paxillin (Norman et al., 1998). Finally, FAK, which binds directly to paxillin through the LD2 and LD4 motifs (Turner et al., 1999), associates with another regulator of p21 GTPase function, the rho GAP, GRAF (Hildebrand et al., 1996). Thus, paxillin is uniquely positioned to function as a molecular scaffold at the plasma membrane. Its NH_2 terminus–containing LD motifs can potentially position these regulatory and structural proteins in close proximity to each other to facilitate the efficient conversion of environmental signaling cues, transduced through integrin engagement with the extracellular matrix, into the localized changes in the actin cytoskeleton required for a variety of normal cellular processes including cell motility and proliferation.

Our observations that certain actopaxin mutants—namely the PBS mutant and the COOH terminus—interfere with the ability of cells to adhere and spread on the extracellular matrix indicate a likely role for actopaxin in these integrin-mediated events. We speculate that the actopaxin PBS mutant interferes with the adhesion process because it is unable to localize to the plasma membrane via an association with paxillin. As a result, the mutant protein

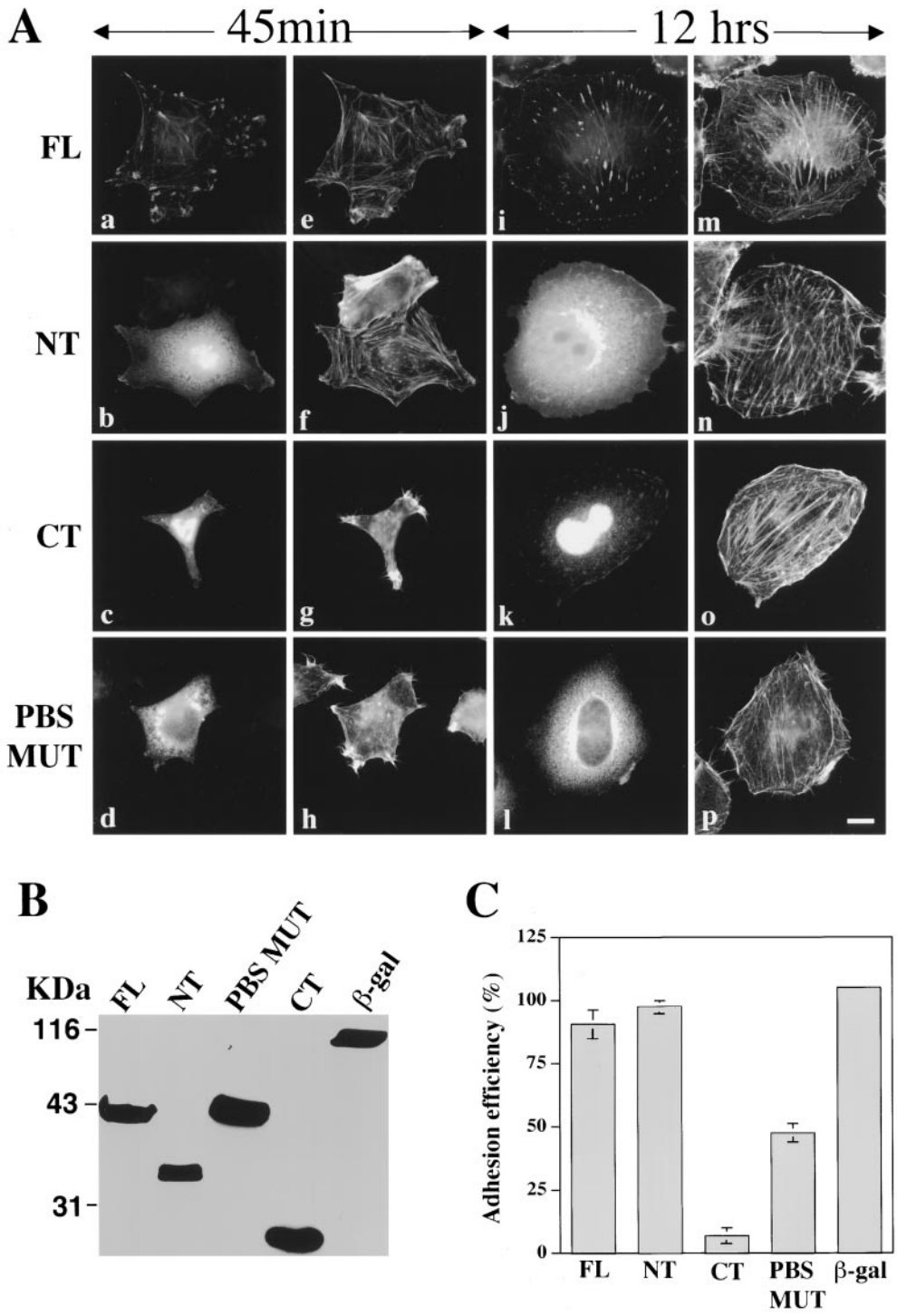

Figure 9. Actopaxin mediates adhesion and spreading of HeLa cells. (A) HeLa cells transfected with Xpress-actopaxin constructs were plated on collagen type I–coated slips for 45 min or for 12 h. For each time point, slips were fixed and processed for immunofluorescence with Xpress antibody. The wild-type full-length actopaxin (FL) localizes efficiently to focal adhesions at both time points (a and i). The COOH terminus actopaxin (CT) localizes weakly to focal adhesions at later time points (c and k). Both the NH_2 terminus (NT) and the PBS mutant (PBS MUT) failed to target to focal adhesions (b–j and d–l). Dual labeling of actin in these cells with rhodamine phalloidin indicates that overexpression of the CT or the PBS mutants results in a partial reduction in actin filament content during the initial phases of cell spreading (g and h). (B) Protein expression of the actopaxin constructs and β-galactosidase control. 50 μg of total cell lysates for each construct of actopaxin transfectants were run on SDS-PAGE, transferred to nitrocellulose, and probed with Xpress antibody. (C) Adhesion and spreading efficiency of HeLa cells expressing wild-type full-length, NH_2 terminus, COOH terminus, PBS mutant actopaxin, or β-galactosidase. Data represent the mean ± SD of three experiments. Bar, 5 μm.

likely sequesters in the cytosol other actopaxin-binding proteins important to the adhesion process. In contrast, the COOH terminus, which can bind paxillin but not actin (Goldsmith et al., 1997; Banuelos et al., 1998; our unpublished observations), may inhibit spreading by displacing endogenous actopaxin from newly forming focal complexes and/or adhesions, thereby preventing efficient organization of the actin cytoskeleton at these sites. This hypothesis is supported by our observations indicating that, although paxillin is efficiently localized to focal adhesions, the actin cytoskeleton is less organized in cells expressing the actopaxin COOH terminus or PBS mutants. However, it should be noted that the overall organization of the actin cytoskeleton in cells expressing these two mutants appears normal several hours after plating. This likely reflects the fact that there are several additional proteins such as talin and vinculin that can function to link actin to the focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996) and also may indicate that actopaxin functions primarily in cytoskeleton assembly only during the early phases of cell spreading or during other dynamic events such as cell migration. Finally, our data do not preclude the possibility

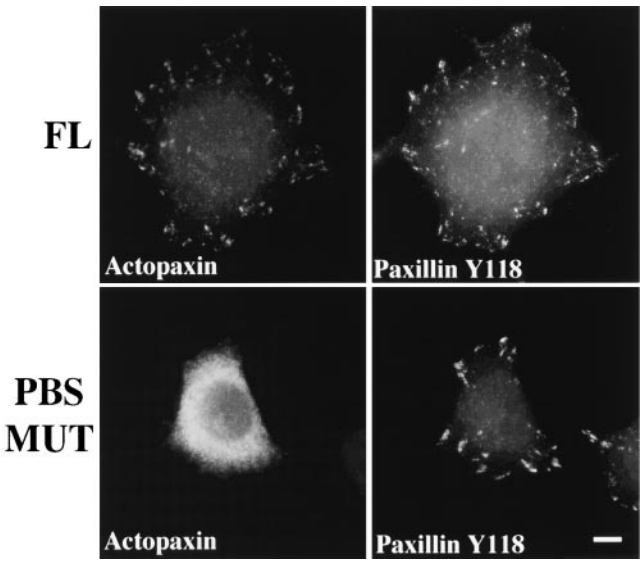

Figure 10. Ectopic expression of wild-type full-length actopaxin or actopaxin PBS mutant has no effect on paxillin localization to focal adhesions during spreading on collagen. HeLa cells transfected with Xpress-actopaxin constructs were plated on collagen type I–coated coverslips. Coverslips were fixed and stained for immunofluorescence with Xpress and paxillin Y118 antibodies. FL, full-length actopaxin; PBSMUT, actopaxin PBS mutant. Bar, 5 μm.

that actopaxin may influence integrin binding to the extracellular matrix as previously demonstrated for the LIM domains of paxillin (Brown et al., 1998b). Future experiments will be directed towards examining these various potential mechanisms of actopaxin action.

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