Molecular Dissection of Actopaxin-Integrin-linked Kinase-Paxillin Interactions and Their Role in Subcellular Localization*

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Actopaxin is a focal adhesion adapter protein involved in integrin signaling. We have recently reported that the paxillin LD1 motif acts as a binding interface for both the actin-binding protein actopaxin and the serine/threonine integrin-linked kinase (ILK). In this report we demonstrate the direct association between actopaxin and ILK and dissect the role of the respective interactions in their subcellular localization. Co-immunoprecipitation experiments were employed to map the binding sites on ILK and actopaxin. ILK binds to the CH2 domain of actopaxin. However, an actopaxin CH2 domain mutant defective for paxillin binding (paxillin binding subdomain mutant) retains the capacity to bind ILK, indicating that paxillin and ILK binding sites on actopaxin are distinct. Actopaxin binds to the C terminus of ILK. Despite the direct binding between actopaxin and ILK, mutation analysis confirmed a primary role for paxillin in their localization to focal adhesions. Interestingly, an ILK mutant (E359K) that was previously reported to act as dominant negative for ILK function was unable to bind actopaxin or paxillin and failed to localize to focal adhesions. This mutant also exhibited in vitro kinase activity comparable with wild-type ILK. Taken together, these data suggest that normal ILK signaling is dependent on efficient localization involving multiple protein interactions.

Cell adhesion to the extracellular matrix is critical for many physiologic processes such as adhesion, spreading, and migration (1) and is mediated primarily by integrins (2–4). Engagement of integrin molecules on the cell surface with the extracellular matrix is accompanied by the recruitment of numerous cytoskeletal and signaling proteins to the cytoplasmic face of these attachment sites, resulting in the formation of structures called focal adhesions. These protein complexes coordinate integrin-mediated signal transduction associated with cell motility, gene expression, and cell proliferation.

Paxillin is a multidomain adapter protein localized to focal adhesions that functions as a molecular scaffold to facilitate signaling (5, 6). The N terminus of paxillin contains five LD motifs that are highly conserved between species and between other paxillin family members (7, 8). These leucine-rich repeats serve as binding sites for the cytoskeletal proteins actopaxin and vinculin (7, 9), as well as for the tyrosine kinase FAK† (7). The LD4 motif also links paxillin to the p21-activated kinase through the Arf GTPase-activating protein paxillin kinase linker and the Rac guanine nucleotide exchange factor p21-activated kinase interacting exchange factor/clone out of library (10). Recently we also demonstrated that the LD1 motif binds directly to the serine/threonine integrin-linked kinase (ILK) (11).

Actopaxin localizes to focal adhesions and binds actin in addition to binding paxillin (9). We have previously shown that paxillin binding is necessary for actopaxin recruitment to focal adhesions (9). In addition, ectopic expression of a paxillin binding-defective actopaxin mutant (paxillin binding subdomain (PBS) mutant) in HeLa cells results in a substantial reduction in cell adhesion/spreading on collagen, suggesting an important role for actopaxin-paxillin interactions in integrin-dependent remodeling of the actin cytoskeleton (9). Actopaxin was also identified independently in a data base screen for actin-binding proteins and named parvin, defining a family of proteins with three members (α, β, and γ) (12).

The ILK is a serine/threonine kinase that was originally identified in a yeast two-hybrid screen for β3 integrin cytoplasmic tail binding proteins (13). The N terminus of ILK has been shown to interact with the LIM-only adapter protein PINCH (14, 15), whereas the C terminus of ILK, which contains the kinase catalytic domain, was shown to interact with β integrin (13) and paxillin (11). ILK kinase activity has been implicated in growth factor signaling (13, 16, 17), as well as in integrin-mediated cellular processes including regulation of cell adhesion (13), fibronectin matrix assembly (18), cell adhesion-dependent cell cycle progression (19, 20), and myogenic differentiation (21). ILK contains a PBS in the C terminus region, and ILK PBS mutants fail to localize to focal adhesions, suggesting an important role for ILK-paxillin association in focal adhesion targeting of ILK and in the role of ILK in integrin-mediated signal transduction events (11).

An interaction between ILK and two actopaxin family members (human actopaxin/a-parvin/CH-ILKBP and β-parvin/affixin) has recently been described (22, 23). However, in these studies their interaction with paxillin was not reported. Consequently, the defects in cell adhesion/spreading caused by CH-ILKBP or affixin mutants were attributed to perturbation of their associations with ILK.

In this study, we confirm a direct association of actopaxin

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† The abbreviations used are: FAK, focal adhesion kinase; aa, amino acid; GFP, green fluorescent protein; CH, calponin homology; PBS, paxillin binding subdomain; ILK, integrin-linked kinase; ILKBP, ILK-binding protein; GST, glutathione S-transferase; MBP, myelin basic protein.
with ILK and further examine a role for paxillin binding in actopaxin and ILK subcellular localization. We show that the association with paxillin is essential for actopaxin and ILK targeting to focal adhesions. Additionally we demonstrate that the “kinase-dead” ILK (E359K) mutant, previously shown to act as a “dominant negative” in several cellular processes, retains wild-type kinase activity as recently reported (23) but fails to bind to paxillin or actopaxin in vivo and also fails to localize to focal adhesions. Taken together these results suggest that correct subcellular localization of ILK through an intact paxillin-actopaxin-ILK protein assembly is likely to impact significantly on normal ILK signaling.

MATERIALS AND METHODS

Antibodies—Polyclonal actopaxin antibody has been described previously (9). Mouse monoclonal ILK antibody (clone 3) was generated in collaboration with Transduction Laboratories (Lexington, KY) and was previously characterized (11). Paxillin monoclonal antibody (clone 349) was also from Transduction Laboratories; paxillin phospho-Y118 polyclonal antibody was a gift from Dr. Erik Schaeffer (BIOSOURCE International (Camarillo, CA)); α-actinin and vinculin monoclonal antibodies were from Sigma; Xpress monoclonal antibody was from Invitrogen (Carlsbad, CA), and GFP rabbit polyclonal antibody was a generous gift of Dr. P. Silver (Dana Farber Cancer Institute, Boston, MA).

Cell Culture and Transfection—Rat aortic smooth muscle cells, rat embryo fibroblasts (REF-52), and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech, Washington, D, C.) supplemented with 10% (v/v) fetal bovine serum and 50 units/ml penicillin-streptomycin and 50 units/ml streptomycin (Sigma). Chinese hamster ovary (CHO-K1) cells were cultured in modified Ham’s F-12 medium (Mediatech) supplemented with 10% (v/v) heat-inactivated, certified fetal bovine serum and 50 units/ml penicillin-50 μg/ml streptomycin at 37 °C in a humidified chamber with 5% CO2. Transfection of HeLa cells was performed with Fugene 6 (Roche Molecular Biochemicals) following the instructions of the manufacturer.

DNA Constructs and Mutagenesis—GFPILK containing a deletion of the first ankyrin repeat (GFPILKΔAnK1) (aa 51–452) (Fig. 3) was generated by PCR using rat ILK as template and Pfu polymerase (Stratagene), followed by subcloning of the PCR product into the EcoRI site of the EGF-C2 vector (CLONTECH). The Xpress-ILK N terminus (aa 1–189) or Xpress-ILK C terminus (aa 190–452) (Fig. 3) was similarly generated by PCR and subcloning of the PCR products into the EcoRI site of the pcDNA3.1HisC vector (Invitrogen). The GFPILKE359K and Xpress-actopaxin F271D mutants were generated with the Quik Change Mutagenesis kit (Stratagene) using the rat GFPILK and Xpress-actopaxin as template, respectively, according to the instructions of the manufacturer. All constructs were sequenced on both strands (BioResource Center, Cornell University, Ithaca, NY). All other ILK or actopaxin constructs were previously described (9, 11).

Preparation of Fusion Proteins and Binding Assays—Individual glutathione S-transferase (GST) fusion proteins of the GST vector, paxillin LD1 motif (aa 1–20), and full-length actopaxin (aa 1–372) were expressed in Escherichia coli (BL21) and purified on glutathione-agarose beads as described previously (10).

For in vitro binding experiments, GST, GST-paxillin LD1, or GST-actopaxin fusion proteins were incubated with 35S-labeled paxillin or actopaxin in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2 mM sodium pyrophosphate, 25 mM sodium β-glycerophosphate, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM p-nitrophenylphosphate, and immunoprecipitation with GFPILK antibody were performed. After four washes with lysis buffer and once with kinase buffer (50 mM Hepes, pH 7.0, 10 mM MnCl2, 10 mM MgOAc, 2 mM NaF, 1 mM Na3VO4), the immunoprecipitates were subjected to protein kinase assays in 20 μl of kinase reaction buffer containing 10 μM of [γ-32P]ATP and 10 μg of myelin basic protein (MBP) as substrate. After incubation for 20 min at room temperature with intermittent mixing, the reactions were resolved on 10% SDS-PAGE. The gel was stained with Coomassie Blue to visualize MBP, dried on paper, and exposed against film for 1 h.

Immunofluorescence Microscopy—Indirect immunofluorescence analysis was performed as previously described (7) with the modification that antibody dilutions were made in Western block buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.2% Tween 20, 3% (w/v) bovine serum albumin). Photographs were taken on a digital SPOTTMRT camera (Diagnostic Instruments Inc, Sterling Heights, MI). Images were processed using Adobe Photoshop 3.0.5.

RESULTS

Association of Actopaxin and ILK in Vitro—We previously described direct interactions between the paxillin LD1 motif and both actopaxin and ILK using GST-paxillin LD motif fusion proteins and 35S-labeled Xpress-tagged actopaxin or Xpress-tagged ILK (9, 11). During the course of these experiments, a GST-actopaxin fusion protein was also tested for its ability to bind 35S-labeled Xpress-ILK. As illustrated in Fig. 1, A and B, Xpress-ILK, in addition to binding paxillin LD1 motif, also bound efficiently to GST-actopaxin, thus indicating a direct association between these two proteins.

Our previous characterization of paxillin interactions with actopaxin and ILK revealed that actopaxin binds to both paxillin LD1 and LD4, whereas ILK binds only to LD1 motif in vitro. The observed direct association of actopaxin and ILK in vitro (Fig. 1, A and B) urged us to examine if ILK still binds exclusively to the paxillin LD1 motif when incubated with cell lysates. Each paxillin GST-LD motif (Fig. 1C) was incubated with rat aortic smooth muscle cell lysates in a precipitation binding experiment followed by Western blotting with ILK antibody. In this case, ILK co-precipitated with both the LD1 and LD4 motif (Fig. 1D). Reprobing of the blot with α-actinin antibody revealed that α-actinin was unable to bind any paxillin LD motif, whereas reprobing with actopaxin antibody confirmed binding to both LD1 and LD4 (9) and data not shown). These results demonstrate the capacity of ILK to bind paxillin both directly and indirectly, potentially through association with actopaxin.

Actopaxin Co-precipitates with ILK in Vivo—To evaluate further the interaction between actopaxin, ILK, and paxillin in vivo, co-immunoprecipitation experiments were performed using lysates from several fibroblast or epithelial cell lines including rat aortic smooth muscle (Fig. 2) and E17-18, CHO-K1 (data not shown). Total lysates from these cell lines were incubated with actopaxin antibody or with control rabbit IgG. Immunoprecipitates were resolved by SDS-PAGE, and Western blots were probed sequentially with ILK, paxillin, and α-actinin antibodies. ILK was strongly co-immunoprecipitated with the actopaxin antibody from all lysates, as was paxillin, whereas α-actinin failed to bind (Fig. 2). In addition, the co-precipitation of actopaxin and paxillin with ILK was retained in lysates from rat aortic smooth muscle cells maintained in suspension for 2 h (Fig. 2), indicating that the actopaxin-ILK-paxillin associations are not adhesion-dependent.

Actopaxin-ILK-Paxillin

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Actopaxin Binds to the ILK C Terminus—To determine the region of ILK required for binding to actopaxin, we performed co-transfection experiments in HeLa cells using GFP-actopaxin with Xpress-ILK (aa 1–452), Xpress-ILK N terminus (aa 1–189), or Xpress-ILK C terminus (aa 190–452) (Fig. 3). Following transfection, cells were lysed in co-immunoprecipitation buffer, and immunoprecipitation using either GFP or control rabbit IgG antibodies was performed. Both endogenous ILK and Xpress-ILK, as well as the Xpress-ILK C terminus, were co-precipitated with GFP-actopaxin (Fig. 4). Paxillin was also co-precipitated under these conditions. A reprobe of the blot with α-actinin showed no binding to the immunoprecipitates, thus confirming the specificity of the associations. In contrast, Xpress-ILK N terminus failed to co-precipitate with actopaxin (data not shown). Taken together these data suggest that the C-terminal domain of ILK contains the binding site for actopaxin in addition to binding sites for paxillin (11) and β1 integrin (13).

The ILK PBS Mutant Binds Actopaxin in Vitro but Not in Vivo—We described previously the presence of a PBS within the C terminus of ILK. Mutations within the PBS of ILK abrogated its association with paxillin in vitro (11). To assess the ability of the ILK PBS mutant to bind actopaxin, we performed both in vitro and in vivo binding experiments. For the in vitro experiments, ILK PBS mutant was generated by coupled transcription-translation as [35S]methionine-labeled protein and used in binding reactions with GST, paxillin GST-LD1 motif, and GST-actopaxin. The ILK PBS mutant was able to bind GST-actopaxin (Fig. 5A), although less efficiently than the wild-type ILK (Fig. 1), thus indicating that actopaxin and paxillin binding sites are distinct. In contrast, when co-precipitation binding experiments were performed with the actopaxin and ILK PBS mutant proteins were expressed in vivo, we were not co-precipitate actopaxin with the ILK PBS mutant (Fig. 5B). Thus, although actopaxin is capable of binding in vitro to an ILK mutant defective for paxillin binding, the reduced affinity of this interaction precludes a stable association between ILK and actopaxin in vivo.

Additionally, we performed binding experiments with a GFP-ILK mutant lacking the first N-terminal ankyrin repeat, a domain previously shown to be critical for PINCH binding (14). Interestingly the ILKΔANK1 mutant retained the capacity to bind actopaxin but failed to efficiently co-precipitate paxillin (Fig. 5B). Although this result provides further evidence that the paxillin and actopaxin binding sites on ILK are distinct, it also suggests that certain ILK mutants may exhibit more global perturbations in structure.

ILK Binds to a Region of the Actopaxin C Terminus That Is Distinct from the Paxillin Binding Site—We have previously mapped the paxillin binding site on actopaxin to a PBS motif (9) in co-transfection experiments with GFP-ILK wild-type. Western blot analysis of GFP immunoprecipitates with actopaxin CH2 domain are separable, we used the Xpress-actopaxin PBS mutant that is incapable of binding paxillin in vitro (9) in co-transfection experiments with GFP-ILK wild-type. Western blot analysis of GFP immunoprecipitates with Xpress Ab showed that GFP-ILK binds as efficiently to the actopaxin PBS mutant as to actopaxin wild-type (Fig. 6B).
Reprobing of the same blot with paxillin showed that co-immunoprecipitation of endogenous paxillin with the GFP-ILK wild-type/Xpress-actopaxin PBS mutant is substantially reduced as compared with GFP-ILK wild-type/Xpress-actopaxin wild-type precipitates and likely represents the fraction of paxillin binding directly to ILK. These results indicate that the ILK and paxillin binding sites on actopaxin are distinct.

A recent report indicated that a point mutant (F271D) of CH-ILKBp (which is identical to human actopaxin/α-parvin) is incapable of binding ILK in vivo and of localizing to focal adhesions in rat mesangial cells (22). We generated the same mutation in rat actopaxin cDNA and tested its capacity to localize to focal adhesions and to bind paxillin and ILK. In our hands, actopaxin F271D localizes to focal adhesions when it is transfected into HeLa cells. In certain cases robust localization was observed (Fig. 7B, a and b) whereas in others localization was attenuated (asterisks in Fig. 7B, c and d). Co-precipitation experiments showed that the actopaxin F271D mutant binds at wild type levels to ILK (Fig. 7A). However, the amount of paxillin co-precipitated in these experiments was substantially reduced to a level similar to that seen with the ILK-actopaxin PBS mutant co-precipitation experiments (see Fig. 6B and data not shown). These data indicate that the reduced overall efficiency of localization of the F271D actopaxin mutant is likely due to perturbation of paxillin, rather than ILK binding.
Actopaxin PBS Mutant Transfectants Can Be Partially Recruited to Focal Adhesions When They Are Co-transfected with ILK—We have previously shown that an actopaxin PBS mutant is unable to target to focal adhesions, as a result of its inability to bind to paxillin (9). Nevertheless, the biochemical data presented above suggest that an ILK-actopaxin interaction may be important for the subcellular localization of these two proteins. Thus, we examined the subcellular distribution of ILK and actopaxin wild-type and mutant proteins expressed in HeLa cells. Both actopaxin wild-type and ILK wild-type localized efficiently to focal adhesions when they were transfected as single constructs, or if they were co-transfected into HeLa cells (Fig. 8A, a and b). In contrast, the actopaxin PBS mutant or the ILK PBS mutant when transfected separately remained cytoplasmic, as we previously reported (9, 11) (and data not shown). Additionally, as expected both the actopaxin PBS mutant and the ILK PBS mutant proteins remained cytoplasmic when co-transfected (Fig. 8A, c and d). Although the ILKΔANK1 mutant is capable of binding actopaxin in vivo (Fig. 5B), it is incapable of localizing to focal adhesions even in the presence of exogenous actopaxin (Fig. 8A, e and f) confirming previous reports (14) (Fig. 8A). However, in co-transfection experiments of GFP-ILK with Xpress-actopaxin PBS mutant, the actopaxin PBS mutant protein was able to localize to focal adhesions in ~50% of transfectants (results of three independent experiments) (Fig. 8, A, g and h, and B). Taken together these data suggest that while the primary mechanism of recruitment of actopaxin and ILK to focal adhesions is through their direct binding to paxillin, alternative mechanisms may be utilized under certain experimental or physiologic conditions.

The ILK E359K Mutant Is Incapable of Binding Either Actopaxin or Paxillin and of Localizing to Focal Adhesions—The ILK E359K mutant has previously been described to function as a kinase-dead form of ILK and to act as a dominant negative for ILK signaling in the context of cell adhesion, fibronectin matrix assembly, cell cycle progression, and myogenic differentiation (13, 16, 18, 21). We have generated the kinase-deficient ILK mutant (E359K) and tested its ability to interact with actopaxin and paxillin by co-transfection experiments of GFP-ILK E359K with Xpress-actopaxin. In contrast to wild-type ILK, which co-precipitated both actopaxin and paxillin, no binding of these proteins to the ILK E359K mutant was observed (Fig. 9A). Furthermore, immunofluorescence staining of ILK E359K transfected cells showed that the ILK E359K mutant cannot target to focal adhesions but remains cytosolic (Fig. 9B). Finally, it has been suggested recently that the ILK E359K mutant may retain kinase activity (23). The kinase activity of the ILK E359K mutant relative to the ILK PBS mutant and the ILK wild-type was examined. GFP vector, GFP-ILK, GFP-ILK E359K, and GFP-ILK PBS mutant were transfected into HeLa cells, and kinase activities of the GFP immunoprecipitates were assayed using MBP as a substrate (Fig. 9C). Western blots with equal amounts of lysates from the transfectants were...
also prepared to determine the amount of exogenous GFP-ILK among transfectants (Fig. 9D). From both experiments we determine there are no significant differences between the basal kinase activity of the wild-type ILK and the E359K or PBS mutants under these assay conditions. Taken together these data suggest that the “kinase-deficient” ILK E359K mutant may exhibit its dominant negative phenotype as a result of inappropriate subcellular localization rather than as a consequence of defective kinase activity.

**DISCUSSION**

We have previously reported that paxillin through its LD1 motif binds directly to the focal adhesion proteins actopaxin (9) and ILK (11). Recently, actopaxin family members have been isolated as ILK-interacting proteins in yeast two-hybrid screens and named CH-ILKBP (identical to human actopaxin/H9251-parvin) (22) and affixin (identical to H9252-parvin) (23). In the present study we confirm that actopaxin and ILK can associate directly and provide further evidence for the importance of paxillin binding in their efficient localization at focal adhesions. Furthermore, we present evidence that the dominant negative effects of the ILK E359K mutant, may be due to a deficiency in paxillin and actopaxin binding and thus loss of

**FIG. 7.** The actopaxin F271D mutant is capable of localizing to focal adhesions and binding ILK in vivo. A, HeLa cells were co-transfected with GFP-ILK and Xpress-actopaxin, Xpress-actopaxin-F271D, or Xpress-actopaxin PBS mutant. Immunoprecipitations with either GFP or control IgG antibodies followed by Western blotting with Xpress, GFP, and α-actinin antibodies were performed. All Xpress-actopaxin constructs bind GFP-ILK. B, HeLa cells transfected with Xpress-actopaxin F271D were plated on fibronectin-coated coverslips and stained with Xpress Ab (a, c) and rhodamine-phalloidin (b) to visualize actin stress fibers or co-stained with paxillin Y118 (d). Note the variability in focal adhesion targeting of the actopaxin F271D mutant. Transfected cells are marked with an asterisk in c. Bar, 5 μm.

**FIG. 8.** A, HeLa cells were co-transfected with GFP-paxillin/Xpress-actopaxin (a and b), GFP-ILK PBS mutant/Xpress-actopaxin PBS mutant (c and d), GFP-ILKΔANK1/Xpress-actopaxin (e and f), or GFP-ILK/Xpress-actopaxin PBS mutant (g and h). 24 h post-transfection the cells were replated on fibronectin-coated slips, and 16 h later the cells were fixed and processed for immunofluorescence with Xpress antibody and rhodamine-conjugated anti-mouse IgG. Bar, 5 μm. B, bar graph representing the percentage of cells exhibiting actopaxin localization to focal adhesions. Bars represent the mean of three independent experiments.
SDS-PAGE followed by Western blotting with ILK antibody. Total lysates from the transfectants used in paxillin, and GFP antibodies. Subjected to SDS-PAGE, followed by Western blotting with Xpress, GFP and control rabbit IgG immunoprecipitations were performed and post-transfection cells were lysed in co-immunoprecipitation buffer. Actopaxin or with GFP-ILK E359K/Xpress-actopaxin constructs. 24 h post-transfection cells were lysed in modified phosphate-buffered saline (PBS) mutant. 24 h post-transfection cells were lysed in modified PBS mutant. GFP-immunoprecipitates using MBP as an exogenous substrate. Reaction mixtures were subjected to SDS-PAGE followed by Coomassie Blue staining of the gel and autoradiography. In vitro kinase assays were performed on the kinase activity.

As is the case with other paxillin LD motif binding proteins such as vinculin and FAK, the C-terminal domains of actopaxin and ILK contain a PBS. Mutation of either the actopaxin or ILK PBS eliminates paxillin binding and also the ability of either actopaxin or ILK to localize to focal adhesions (9, 11). Interestingly, and consistent with recent reports (22, 23), we find that ILK and actopaxin also interact directly through their respective C-terminal domains. However, although ILK, like paxillin, binds to the second CH domain of actopaxin, its binding is unaffected by an actopaxin PBS mutation, thus demonstrating that paxillin and ILK binding sites on actopaxin are distinct. Similarly, ILK PBS mutants retain the capacity to bind actopaxin in vitro, indicating that the paxillin and actopaxin binding sites within the ILK C terminus are also distinct. However, the ILK PBS mutant was unable to bind actopaxin in vivo, suggesting that the two binding sites are closely juxtaposed and that the PBS mutation is sufficient to interfere with optimal folding of the adjacent actopaxin binding site. Additionally, certain mutations within the ILK molecule, such as the E359K mutant (see below) and the ΔANK1 mutant, appear to exert more global effects on protein folding. The N-terminal ANK1 repeat has previously been identified as the PINCH binding site (14). However, the ΔANK1 ILK mutant has also lost the capacity to bind paxillin, while retaining functional actopaxin binding. Consistent with previous reports (14) the ΔANK1 ILK mutant remained cytosolic when transfected into HeLa cells, thus indicating that robust actopaxin binding is insufficient for ILK recruitment to focal adhesions. Whether ILK requires both PINCH and paxillin binding for efficient targeting remains to be determined.

Our previous studies have suggested that paxillin binding is essential for normal localization of actopaxin to focal adhesions. Recently, however, it was reported that a point mutation (F271D) of CH-ILKBP (identical to human actopaxin) abrogated the CH-ILKBP-ILK association in vitro and rendered the protein incapable of localizing to focal adhesions when expressed in rat mesangial cells. These data suggested that CH-ILKBP (actopaxin) is recruited to focal adhesions through its association with ILK (22). We created the same mutation in rat actopaxin and found that the actopaxin F271D mutant binds ILK in vivo as efficiently as the wild-type actopaxin or its PBS mutant. In contrast, co-precipitation of paxillin was substantially reduced to a level similar to that seen with the ILK-actopaxin PBS mutant combination. Additionally, the actopaxin F271D mutant localized to focal adhesions when introduced into HeLa cells albeit with reduced efficiency compared with the wild-type protein. Taken together, these results confirm that mutation of residue Phe-271 compromises actopaxin targeting to focal adhesions, but that this is likely due to perturbation of paxillin binding rather than the actopaxin-ILK association. Indeed Phe-271 is only two residues upstream of the minimal PBS domain (9).

These data reinforce a primary role for paxillin in the recruitment of actopaxin to focal adhesions. However, one result that is in potential conflict with this model is the ability of an actopaxin PBS mutant to localize to focal adhesions in 50% of the cells when it is co-transfected with ILK. Although in the absence of ILK overexpression the actopaxin PBS mutant never localizes to focal adhesions (Figs. 8 and 9), this result suggests that under certain physiologic conditions ILK binding alone may be sufficient for actopaxin recruitment. It should be noted, however, that this does not preclude a role for paxillin in this particular scenario, because ILK targeting is dependent on paxillin. Thus, although actopaxin can bind directly to both the focal adhesion localization, as opposed to a defect in kinase activity.

Fig. 9. A, the ILK E359K mutant is incapable of binding actopaxin and paxillin. HeLa cells were co-transfected with GFP-ILK/Xpress-actopaxin or with GFP-ILK E359K/Xpress-actopaxin constructs. 24 h post-transfection cells were lysed in co-immunoprecipitation buffer. GFP and control rabbit IgG immunoprecipitations were performed and subjected to SDS-PAGE, followed by Western blotting with Xpress, paxillin, and GFP antibodies. Bar, 5 μm. C, HeLa cells were transfected with GFP, GFP-ILK wild-type, GFP-ILK E359K, or GFP-ILK PBS mutant. 24 h post-transfection cells were lysed in modified radioimmune precipitation buffer, and immunoprecipitations with GFP antibody were performed. In vitro kinase assays were performed on the GFP-immunoprecipitates using MBP as an exogenous substrate. Reaction mixtures were subjected to SDS-PAGE followed by Western blotting with ILK antibody.
LD1 and LD4 motifs of paxillin, it may also interact indirectly with paxillin via ILK binding to LD1. Similarly, we have found that ILK can interact indirectly with the paxillin LD4 motif, possibly through association with actopaxin. How the cell utilizes the differential binding capacity of the paxillin LD motifs (6) to facilitate intracellular signaling represents an important area for future study.

ILK kinase activity has been implicated in the regulation of multiple physiologic processes including cell adhesion/transformation (13), fibronectin-matrix assembly (18), E-cadherin expression (17), phosphorylation of PKB/Akt (16), cell survival (24), and myogenic differentiation (21). These assignments were based primarily on the effects of overexpression of the ILK E359K mutant relative to wild-type ILK. In this study we demonstrate that in precipitation kinase assays the ILK E359K mutant exhibits basal kinase activity comparable with the wild-type protein as reported by others (23). Importantly, however, the ILK E359K mutant is incapable of interacting with actopaxin and paxillin in vivo. This is perhaps not surprising considering the close proximity of the Glu-359 residue to the defined minimal PBS of ILK which starts at Ser-377 (11). Accordingly, the ILK E359K mutant does not localize to focal adhesions when expressed in HeLa cells. Taken together these observations suggest that the dominant negative effects exerted by the ILK E359K mutant may be due to inappropriate subcellular localization as a result of its inability to bind paxillin (and actopaxin), rather than due to alterations in kinase properties per se. Nevertheless, it is likely that correct subcellular localization of ILK is essential for appropriate integrin- and growth factor-mediated stimulation of ILK kinase activity and subsequent downstream signaling events.

Finally, both ILK and actopaxin have homologues in Caenorhabditis elegans (pat-4 and pat-6, respectively) and Droso-phia. A proposed role for these proteins in mediating actin-membrane attachment has been suggested by genetic studies that include mutations/deletions of these homologues. Loss of pat-4 or pat-6 results in phenotypes that resemble the elimination of α or β integrin homologues (pat-2 and pat-3, respectively) and are characterized by defects in muscle cell body and dense bodies (25–27). In addition, mutations in Drosophila ILK results in detachment of actin filaments from the membrane at the muscle attachment sites. Again this phenotype is comparable with that observed following loss of integrin function and suggests a primary structural role for ILK and actopaxin in linking integrins to the actin cytoskeleton (28). Paxillin is similarly localized to skeletal muscle myotendinous junctions and smooth muscle dense plaques in higher eukaryotes (29). A paxillin homologue has recently been described in Drosophila where its expression pattern coincides with sites of high inte-grin mRNA levels (30). Importantly, the domain structure of paxillin, including the LIM domains and LD motifs, is highly conserved across species. Thus, in addition to potentially facilitating integrin-mediated signal transduction, the paxillin-actopaxin-ILK associations discussed in this report are likely to be of fundamental importance in the structural organization of extracellular matrix-actin interactions and thus normal muscle function.

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