αPIX Associates with Calpain 4, the Small Subunit of Calpain, and Has a Dual Role in Integrin-mediated Cell Spreading*

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Binding of integrins to the extracellular matrix results in actin cytoskeletal rearrangements, e.g., during cell spreading, by regulating the activity of Rho GTPases. We have shown previously that αPIX (Cool-2 or ARHGEF6), a Rac1/Cdc42-specific guanine nucleotide exchange factor (GEF), binds to β-parvin/affixin and co-localizes with integrin-linked kinase in actively spreading cells, suggesting that αPIX is involved in integrin-induced signaling leading to activation of Rac1/Cdc42. Here we report calpain 4, the small subunit of the pro-enzymes μ-calpain and m-calpain, as a novel binding partner of αPIX. This association was identified by the CytoTrap system and confirmed by communoprecipitation and glutathione S-transferase pull-down assays. The αPIX triple domain SH3-DH-PH was found to be required for calpain 4 binding. During integrin-dependent spreading of CHO-K1 cells, αPIX colocalized with μ- and m-calpain, integrin-linked kinase, and β1 integrin in early integrin-containing clusters. Overexpression of αPIX wild type but not the GEF-deficient mutant (L386R/L387S) resulted in enhanced formation of characteristic cellular protrusions during cell spreading, suggesting that αPIX GEF activity is necessary for this specific actin cytoskeletal reorganization. The calpain inhibitors calpeptin and calpain inhibitor IV significantly inhibited integrin-dependent cell spreading. However, concomitant overexpression of αPIX wild type or the L386R/L387S mutant restored cell spreading. Together, these data suggest that αPIX is a component of early integrin clusters and plays a dual role in integrin-dependent cell spreading. Whereas αPIX GEF activity contributes to enhanced formation of cellular protrusions, the GEF-independent association with calpain 4 leads to induction of a yet unknown signaling cascade resulting in cell spreading.

Cell adhesion to the extracellular matrix (ECM) generates various signals that regulate important physiological events including spreading, migration, and growth. All these mechanisms involve changes in organization of the actin cytoskeleton. Interaction of cells with the ECM is mediated by transmembrane receptors termed integrins. Binding of integrins to components of the ECM induces clustering of these receptors that subsequently results in the intracellular recruitment of structural and signaling molecules at the sites of matrix attachment, thereby providing links to the actin cytoskeleton (1, 2).

Upon activation of integrins via ECM engagement, regulation of the actin cytoskeletal dynamics occurs primarily via the Rho family of small GTPases, i.e. Cdc42, Rac, and Rho (3). Rho GTPases function as binary switches that cycle between an active GTP-bound form and an inactive GDP-bound form (4, 5). In particular, activation of Rho increases cell contractility and leads to the formation of focal adhesions and actin stress fibers (3, 6). Cdc42 and Rac activation propagates the formation of filopodia, lamellipodia, and peripheral membrane ruffles as well as focal contacts/complexes (7, 8). The regulated activation of Rho GTPases by growth factor receptors and G protein-coupled receptors has been studied extensively (9). Considerably less is known about how the ECM acts as an insoluble stimulus of the reorganization of the cytoskeleton. Recent studies provide evidence that members of the Rho family of GTPases are activated after signaling through integrins and are involved in integrin-induced cell spreading (10–13). However, the upstream and downstream signaling pathways of Rho GTPases need to be defined in detail.

Activation of Rho GTPases depends on the regulated action of guanine nucleotide exchange factors (GEFs). Vav2, an exchange factor for Rac1, Cdc42, and RhoA (14), has been shown to be necessary for integrin-dependent activation of Rac leading to lamellipodia formation in fibroblasts (15), suggesting that GEFs are also involved in integrin-induced activation of Rho GTPases. αPIX/Cool-2/ARHGEF6, an exchange factor for Rac1 and Cdc42 (16, 17), mediates PAK activation upon cell adhesion to fibronectin (18). Moreover, αPIX stimulates platelet-derived growth factor-induced peripheral spreading of X. nematodes mesoderm aggregates on fibronectin, suggesting a role of αPIX in integrin-mediated cell adhesion and spreading (18). αPIX and its close homologue, βPIX, are part of a large protein complex including PAK, GIT1 (G protein-coupled receptor kinase interactor 1)/p95PKL, and associated proteins implicated in actin cytoskeletal regulation (19–22). Moreover, GIT1/p95PKL is critically involved in the regulation of actin cytoskeletal changes that accompany integrin engagement with the ECM as well as subsequent cell spreading and motility (23).

Other molecules that play a role in integrin-induced signaling are the intracellular, Ca2+-dependent calpain proteases with the two major forms μ-calpain and m-calpain, both consisting of a large catalytic and a small regulatory subunit (24, 25). The 80-kDa catalytic subunits of the μ- and m-forms are encoded by different genes, whereas the 28-kDa regulatory subunit, calpain 4, is common to both forms. Recent data sug-

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The abbreviations used are: ECM, extracellular matrix; GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; PIX, PAK-interacting exchange factor; ILK, integrin-linked kinase; HA, hemagglutinin; GST, glutathione S-transferase; CH, calponin homology; SH, src homology; DH, Dbl homology; PH, pleckstrin homology; GBD, GIT1-binding domain; CC, coiled-coil domain; CHO, Chinese hamster ovary; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.
gest that calpain activity is regulated during signaling pathways leading to cell adhesion, spreading, and motility (26, 27). Moreover, calpain is required for the formation of a new type of integrin complex that induces, by yet unknown means, the generation of focal complexes and adhesions during integrin-mediated cell spreading (28, 29).

Recently, it has been shown that αPIX interacts with the foci adhesion protein β-parvin/affixin (30–32). β-Parvin, integrin-linked kinase (ILK), and β3 integrin assemble in a protein complex that transduces signals from the ECM to intracellular effector proteins (33, 34). The interaction of β-parvin with αPIX and colocalization of αPIX and ILK during cell spreading suggested an involvement of αPIX in integrin-induced signaling, which in turn results in cytoskeletal rearrangements via the GTPases Rac1 and Cdc42 (32, 35). In the present study, we identified calpain 4, the regulatory subunit of both μ- and m-calpain, as an αPIX-interacting protein. We show that αPIX regulates integrin-mediated cell spreading in a GEF-dependent and -independent manner.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening

We used the yeast two-hybrid system CytoTrap (Stratagene), also designated Sos Recruitment System (36), to identify αPIX-interacting proteins. Therefore, αPIX was fused to the N-terminal 1070 residues of human SOS and used as bait. We screened a human fetal brain plasmid cDNA library (Stratagene) with 5.3 × 10⁶ primary colonies and an average insert size of 1.3 kb according to the manufacturer’s instructions. The prey cDNAs were fused to the myristoylation signal of v-Src that anchors the fusion proteins to the plasma membrane. All yeast clones (37). For the CytoTrap screening, the pretransformed cdc25H with pSos-MafB and pMyr-MafB served as a positive (negative control). Only those clones growing on galactose media at the restrictive temperature of 37 °C after 6 days were defined as “true” positive. Cotransformation of the cdc25H yeast strain (see above) was transformed with 3 μg of pMyr-cDNA library plasmids. Resulting transformants were grown for 5 days at 22 °C on selective minimal glucose plates (complete supplement mixture-LU-URA-TRP). After replica plating onto selective minimal galactose plates, a total of 54 colonies of ~2 million transformants showed galactose-dependent growth under restrictive conditions. Plasmid DNA was isolated, transformed into Escherichia coli DH10B, and selected on 50 ng/μl chloramphenicol for the presence of the cDNA insert-containing pMyr plasmid. Protein interactions of putatively positive colonies were confirmed by retransformation of the cdc25H yeast strain with both the cDNA-containing pMyr plasmid and αPIX or empty pGAL4 (negative control). Only those clones growing on galactose media at the restrictive temperature of 37 °C after 6 days were defined as “true” positives. Cotransformation of the cdc25H cells with pSos-MaB and pMyr-target cDNA, pSos and pMyr-target cDNA, pSos-MaB and pMyr-Lamin C, pSos and pMyr, pSos-αPIX and pMyr-Lamin C, and pSos-αPIX and pMyr served as negative controls, whereas cotransformation of the cdc25H cells with pSos-MaB and pMyr-MaB served as a positive control.

In subsequent yeast two-hybrid experiments, different domains of αPIX were tested for interaction with calpain 4, using various bait constructs (see “Plasmid Constructs”) in combination with plasmid pMyr-calpain 4 as prey. Expression of Sos-αPIX fusion proteins was confirmed by immunoblotting of yeast lysates with mouse mononclonal anti-Sos1 antibody (1:400; BD Biosciences) and HRP-conjugated mouse monoclonal antibody (1:5000; Amersham Biosciences). For data base searches, we used the NCBI BLAST Network Service. Deduced protein sequences were searched for functional domains using PROSITE and SMART. GenBank accession numbers are AF207831 and D25304 for the αPIX sequence and NM_001749 for calpain 4.

Plasmid Constructs

αPIX Constructs Used as Bait in CytoTrap—Full-length and various αPIX constructs were established by using specific PCR primers and the KIAA0006 clone as template, and PCR products were ligated unidirectionally into pSos (Stratagene) via Sall and NotI restriction sites.

Generation of N-terminal HA-tagged αPIX Constructs—Various HA-tagged αPIX constructs were generated by using specific PCR primers and the KIAA0006 clone as template, and PCR products were purified and cloned as NotI-EcoRI fragments in eukaryotic expression vector pMT2SM-HA. αPIX ΔDH was generated by PCR-mediated cloning. Two overlapping PCR products lacking the DH domain were amplified and applied to megaprime PCR. The αPIX L386R/L387S construct was generated by PCR-mediated mutagenesis (38) to create two residues abolish GEF activity in αPIX (39) and βPIX (17). Two overlapping cDNA fragments were amplified with the desired mutations and applied to megaprime PCR.

Generation of N-terminal FLAG-tagged Calpain 4 Construct—The calpain 4 coding region was amplified from human testis cDNA (Invitrogen), and the purified PCR product was cloned into “donor vector” pENTR/D-TOPO (Invitrogen) according to the protocol provided. The DNA insert was sequenced for integrity and pENTR/D-TOPO-calpain 4 was used for cloning the calpain 4 coding region into pFLAG-CMV-4-cassetteA (32) via LR reaction according to the manufacturer’s protocol.

Generation of N-terminal GST-tagged Calpain 4 Construct—pENTR/D-TOPO-calpain 4 was used for cloning the coding region of calpain 4 into Gateway pDEST27 vectors (Invitrogen) via LR reaction.

All described constructs were sequenced for integrity, and large and pure amounts of plasmid DNA were prepared by using a plasmid midi or maxi kit (Qiagen). Primer sequences are available on request.

Com Immunoprecipitations

CHO-K1 cells were cultured in 100-mm culture dishes in nutrient mixture F-12 (HAM) containing 10% fetal calf serum and penicillin-streptomycin (Invitrogen). 1.2 × 10⁶ CHO-K1 cells were transfected with pMT2SM-HA or pMT2SM-HA. Escherichia coli DH5α was generated by PCR-mediated cloning. Two

GST Fusion Affinity Precipitations

CHO-K1 cells cotransfected with pDEST27-calpain 4 (5 μg of DNA) and various pMT2SM-HA-αPIX constructs (5 μg of DNA) were lysed with ice-cold lysis buffer, and lysates were clarified by centrifugation for 10 min at 20,000 × g at 4 °C. The supernatants were incubated with 75 μl of GST fusion protein-coupled magnet beads (GSTrap CN1, Amersham Biosciences) and subsequently washed three times with washing buffer (50 mM Tris-HCL, pH 7.5, 250 mM NaCl) and subjected together with total lysates to SDS-PAGE and immunoblot analysis. Proteins were detected using HRP-conjugated murine monoclonal anti-galactose (1:4000; Sigma) or rabbit anti-PIX antibody (1:400; 17) followed by incubation with HRP-conjugated anti-rabbit antibody (1: 5000; Amersham Biosciences).

For affinity precipitations in the presence of calpain inhibitors, cell culture medium was supplemented with Me2SO (control), calpeptin (75 μg/ml in Me2SO), or calpain inhibitor IV (100 μg/ml in Me2SO). Cells were cotransfected with pDEST27-calpain 4 (5 μg of DNA) and various pMT2SM-HA-αPIX constructs (5 μg of DNA) and incubated overnight in supplemented medium. Cells were lysed with ice-cold lysis buffer containing Me2SO (control), calpeptin (75 μg/ml in Me2SO), or calpain inhibitor IV (100 μg/ml in Me2SO).

Cell Plating—Serum-starved CHO-K1 cells were washed with PBS and detached with 0.5% trypsin-EDTA (Invitrogen). Trypsin reaction was stopped using 0.25 mg/ml soybean trypsin inhibitor. To eliminate growth factors, cells were washed once with PBS, once with Puck’s...
saline, and twice with 1% bovine serum albumin in nutrient mixture F-12 (HAM). Finally, cells were resuspended in 0.2% bovine serum albumin in nutrient mixture F-12 (HAM), and 2–3 × 10^5 cells were plated onto fibronectin-covered coverslips. To ensure that cell spreading was indeed fibronectin-induced, we seeded serum-starved CHO-K1 cells onto coverslips coated with fibronectin versus coverslips without fibronectin. Quantification of cell spreading showed that in the case of fibronectin-coated coverslips, 83% of cells could spread, whereas only 22% of cells plated in the absence of fibronectin were able to spread (data not shown). For ectopic expression of various HA-tagged αPIX proteins, CHO-K1 cells were seeded at 1.2 × 10^6 cells/100-mm cell culture dish in complete culture medium. After 15 h, serum was reduced to 0.5%, and 24 h later, cells were transfected with pMT2SM-HA-αPIX wild type, pMT2SM-HA-αPIX (L386R/L387S), or pMT2SM-HA-αPIX ADH (6 μg of DNA each) in the absence of serum according to the manufacturer’s protocol. Cells were incubated overnight at 37 °C in 5% CO2, and prepared for cell spreading experiments on the next day.

**Inhibition of Cell Spreading Using Calpain Inhibitors**—Before plating onto fibronectin-coated coverslips, detached cells were incubated in nutrient mixture F-12 (HAM) supplemented with calpain inhibitor for 30 min. The following inhibitors were tested: the reversible, cell membrane-permeable inhibitor calpeptin (75 μg/ml; Calbiochem), which nucleophilically attacks the active site of m-calpain, and contain 400 bp of the 3’-untranslated region (Fig. 1B). Calpain 4 consists of two domains, the N-terminal glycine-rich domain V, which is often referred to as a hydrophobic domain, and C-terminal domain VI, which contains five EF-hand Ca^{2+}-binding sequences (24). The two cDNAs obtained encode only four EF-hand calcium-binding motifs (Fig. 1B).

To verify the interaction between αPIX and calpain 4 in mammalian cells, we performed communoprecipitation experiments in CHO-K1 cells. αPIX was detected by polyclonal anti-αPIX antibodies (17) that recognize, in addition to αPIX, two isoforms of βPIX (βPIX and β2PIX) (40), a close αPIX homologue. Endogenous αPIX was coimmunoprecipitated with FLAG-calpain 4 (Fig. 1C, top panel, lane 2), but not with empty FLAG-vector control (Fig. 1C, top panel, lane 1). In addition, an in vivo GST pull-down assay was performed using CHO-K1 cells cotransfected with both GST-calpain 4 and wild-type HA-tagged αPIX. As shown in Fig. 2 (middle panel, lanes 1 and 2), GST-calpain 4 fusion protein bound full-length HA-αPIX, whereas GST alone failed to bind αPIX. Together, these data suggest that calpain 4 is a binding partner of αPIX in vivo.

**SH3-DH-Ph Triple Domain of αPIX Is Necessary for Association with Calpain 4**—In GST pull-down experiments, GST-calpain 4 was able to trap all HA-αPIX proteins with the triple domain SH3-DH-PH (Fig. 2, middle panel, lanes 2, 4, 6, and 10), whereas the CH-SH3, DH, DH-PH, and C-terminal GBD-CC domains of αPIX were not able to bind calpain 4 (Fig. 2, middle panel, lanes 12, 14, 16, and 18). Remarkably, an αPIX protein lacking only the DH domain (αPIX ΔDH) showed no affinity to calpain 4 (Fig. 2, middle panel, lane 20). These data suggest that the integrity of the three αPIX domains SH3-DH-PH is necessary for efficient binding of the small subunit of calpain (calpain 4). These data are in line with our findings obtained by the CytoTrap system using various αPIX bait constructs in combination with pMyr-calpain 4 as prey. Again, the three domains SH3-DH-PH of αPIX are required for binding to calpain 4 (data not shown).

**αPIX Localizes in Small Dotted Structures at the Cell Periphery and Enhances the Formation of Cellular Protrusions upon Integrin-induced Cell Spreading**—By immunofluorescence analysis, we examined the effect of wild-type and various mutant HA-tagged αPIX proteins upon integrin-induced cell spreading after 30 min. Overexpression of αPIX wild-type protein caused extensive morphological changes including the formation of various cellular protrusions that represent lamellae sheets (Fig. 3, A1 and A4) with small finger-like protrusions at their end (Fig. 3, A2 and A6). αPIX was located in a punctuated pattern at the cell periphery. These aggregates most likely correspond to the early integrin clusters described previously (29, 41). In contrast, cells expressing a GEF-deficient αPIX mutant (L386R/L387S) or αPIX ADH did not show this characteristic and enhanced formation of protrusions (Fig. 3, B1 and C1). αPIX mutant L386R/L387S was distributed in the cytoplasm as well as at the cell periphery, whereas αPIX ADH localized diffusely in the cytoplasm and was not enriched at the plasma membrane. These data suggest that the GEF activity of αPIX is necessary for the formation of distinct cellular protrusions during integrin-dependent cell spreading. Moreover, the DH domain seems to be required for proper targeting of αPIX to the cell periphery.

**αPIX Colocalizes with the Small and Large Subunits of m- and μ-calpain, ILK, and β1 Integrin in Early Integrin Clusters during Cell Spreading**—To determine the type of αPIX-containing protein aggregates during integrin-dependent cell spreading, we analyzed the colocalization status of ectopically expressed HA-αPIX with endogenous calpain subunits in serum-starved CHO-K1 cells (Fig. 4, A–C). 30 min after seeding on fibronectin, αPIX wild-type-expressing cells formed enhanced lamellipodia-like sheets during cell spreading, and...
**FIG. 1.** αPIX and calpain 4 associate *in vitro*. **A,** αPIX interacts with calpain 4 in the CytoTrap two-hybrid system. Various bait (pSos) and prey (pMyr) plasmids were used for cotransformation of cdc25H yeast cells, representing a positive control (lane 1) and negative controls (lanes 2–7). Five independent cdc25H transformants were spotted on glucose (GLU) medium at 22 °C (left panel) or 37 °C (middle panel) and on galactose (GAL) medium at 37 °C (right panel). Yeast transformants expressing αPIX and calpain 4 amino acids 127–269 grew efficiently on galactose medium at 37 °C (right panel, lane 8).

**B,** domain structure of calpain 4 wild type and two parts identified in the CytoTrap system. Numbers above the domains correspond to the amino acid positions. Calpain 4 consists of two domains, domain V with two glycin-rich regions (dotted boxes) and domain VI comprising five Ca²⁺ binding EF-hand (EFh 1–5) motifs (gray boxes). The two calpain 4 clones encode amino acids 127–269 and amino acids 141–269, respectively, each containing a 3′-untranslated region of ~400 bp (hatched line).

**C,** confirmation of the αPIX-calpain 4 interaction by coimmunoprecipitation. Full-length FLAG-tagged calpain 4 was expressed in CHO-K1 cells and immunoprecipitated with anti-FLAG antibody. αPIX and β1PIX and β2PIX were present in the precipitates from cells transfected with FLAG-calpain 4 (top panel, lane 2), but not from FLAG-vector transfected cells (top panel, lane 1). Expression of endogenous αPIX and two βPIX variants was simultaneously detected by the anti-PIX antibody in total cell lysates (top panel, lanes 3 and 4), and expression of FLAG-calpain 4 was confirmed by immunoblot of total cell lysates (bottom panel, lane 4) and precipitates (bottom panel, lane 2).
αPIX localized in punctual clusters at the cell periphery (Fig. 4, A1, B1, and C1). Staining of endogenous calpain 4 and the large subunits of μ-calpain (calpain 1) and m-calpain (calpain 2) revealed that calpain 4 and calpain 1 were localized around the nucleus (Fig. 4, A2 and B2), whereas calpain 2 was distributed in the cytoplasm in small dots (Fig. 4, C2). Nonetheless, all three proteins colocalized with αPIX in small dots in lamellae sheets or in small finger-like protrusions close to the membrane (Fig. 4, A4, B4, and C4). The presence of both μ- and m-calpain (large and small subunits) in a punctual pattern at the cell periphery suggests that these protein complexes represent initial integrin clusters that form immediately upon integrin-induced signaling (29). αPIX is a novel component of these early clusters and is possibly involved in integrin-mediated activation of Rac1 and/or Cdc42.

To elucidate the composition of these initial integrin clusters, we stained αPIX-overexpressing CHO-K1 cells with various antibodies during integrin-dependent cell spreading. We observed colocalization of αPIX wild type with ILK and β1 integrin in dotted structures at the cell surface (Fig. 4, D4 and E4). In contrast, no colocalization was found for αPIX and paxillin or vinculin in actively spreading cells (Fig. 4, F4 and G4).

Both Wild-type αPIX and the GEF-deficient Mutant (L386R/L387S) Restore Cell Spreading and Associate with Calpain 4 in the Presence of Calpain Inhibitors—To examine whether cell spreading of CHO-K1 cells can be suppressed by inhibition of calpain, we serum-starved CHO-K1 cells and pretreated the cells with various calpain inhibitors and Me2SO, respectively. 77% and 82% of cells incubated with calpeptin or calpain inhibitor IV were unable to form early lamellae protrusions and appeared round (Fig. 5, A and C, untransfected controls, middle and bottom panels). In contrast, 75% of control cells were still able to spread and formed lamellipodia after 30 min (Fig. 5A and C, untransfected controls, top panel). Cells transiently overexpressing αPIX wild type or the L386R/L387S mutant extended membrane protrusions similar to lamellipodia in the

**Fig. 2.** The αPIX SH3-DH-PH triple domain binds calpain 4. The domain structure of various αPIX proteins is shown. CHO-K1 cells were cotransfected with the indicated HA-tagged αPIX constructs and GST-calpain 4. As a control, empty GST-vector was used. The GST-tagged protein complexes were isolated using glutathione-Sepharose beads (GST pull-down) and subjected to immunoblot analysis with anti-HA antibody. GST-calpain 4 was able to bring down all HA-tagged αPIX proteins containing the triple domain SH3-DH-PH (middle panel, lanes 2, 4, 6, 8, and 10). In contrast, no HA-αPIX was precipitated from cells transfected with αPIX constructs lacking at least one of these domains (middle panel, lanes 12, 14, 16, 18, and 20). None of the expressed αPIX proteins was coprecipitated with GST alone (middle panel, lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19). Expression of HA-tagged αPIX proteins in total lysates was confirmed by immunoblot (top panel, lanes 1–20). In precipitates, expression of GST-calpain 4 or GST was detected by reprobing membranes with anti-GST antibody (bottom panel, lanes 1–20).
presence of calpain inhibitor IV or calpeptin, 30 min after seeding on fibronectin (Fig. 5, B1–B4; data not shown). In contrast, cells expressing α/PIX DH appeared round and did not form membrane extensions in the presence of calpain inhibitor IV (Fig. 5, B5 and B6) or calpeptin (data not shown). Quantitative analysis showed that 52–62% of cells expressing α/PIX wild type or L386R/L387S, respectively, could spread in the presence of calpain inhibitors (Fig. 5 C, middle and bottom panels). Conversely, only 24% and 15% of cells expressing the α/PIX DH mutant were able to spread under the respective condition that is comparable to untransfected control cells (Fig. 5 C, middle and bottom panels). When cells were treated only with Me2SO, 76% of α/PIX wild type-overexpressing cells, 73% of α/PIX L386R/L387S-overexpressing cells, and 69% of α/PIX ΔDH-overexpressing cells exhibited a spreading phenotype (Fig. 5C, top panel). The calpain inhibitor calpeptin was known to inhibit both μ- and m-calpain, whereas the specificity of calpain inhibitor IV is restricted to m-calpain. The μ-calpain-specific inhibitor PD 151746 was found not to significantly inhibit integrin-mediated cell spreading because only 31% of the treated cells were unable to spread, which is comparable with 25% of Me2SO-treated control cells (data not shown).

In GST pull-down experiments, GST-calpain 4 was able to bring down HA-αPIX wild type and HA-αPIX L386R/L387S in both the absence and presence of calpain inhibitors (Fig. 5D, middle panel, lanes 1, 2, 4, 5, 7, and 8), whereas α/PIX ΔDH failed to interact with GST-calpain 4 under either of the conditions analyzed (Fig. 5D, middle panel, lanes 3, 6, and 9). This finding is in agreement with the results obtained by GST pull-down experiments shown in Fig. 2 and indicates that calpain inhibitors do not impair the association between α/PIX and calpain 4, an interaction that is likely crucial for spreading of CHO-K1 cells in the presence of calpain inhibitors.

DISCUSSION

In this study we describe a novel interaction between the Rac1/Cdc42-specific guanine nucleotide exchange factor α/PIX and calpain 4, the small subunit of μ/m-calpain. Calpains are Ca2+-dependent cysteine proteases with a large number of substrates including several cytoskeletal proteins and signaling molecules (24).

We showed that α/PIX colocalizes with calpain 4, calpain 1 and 2, ILK, and β1 integrin in small protein aggregates at the cell periphery during cell spreading, whereas no colocalization...
was observed with paxillin and vinculin. Our data suggest that these protein aggregates correspond to the early integrin clusters described by Bialkowska et al. (29), and αPIX provides a direct link between integrin-induced signaling and cytoskeletal reorganization during cell spreading in CHO-K1 cells. However, it has been reported that vinculin is a protein component of the integrin clusters in bovine aortic endothelial cells (29). Possibly, vinculin is recruited to these clusters at a later stage of their development.

Cell spreading is a highly dynamic process. Structural and signaling molecules are required for both assembling and disassembling integrin adhesion complexes and for reorganization.

**FIG. 4.** αPIX colocalizes with calpain 4, calpain 1, calpain 2, ILK, and β1 integrin, but not with paxillin or vinculin in punctual clusters at the cell periphery during cell spreading. Serum-starved CHO-K1 cells expressing HA-tagged αPIX wild type were plated onto fibronectin-coated coverslips to induce integrin-dependent cell spreading. After 30 min, cells were fixed, and HA-αPIX was labeled with anti-HA antibody (A1–G1). Subcellular distribution of endogenous calpain 4 (A2), calpain 1 (B2), calpain 2 (C2), ILK (D2), β1 integrin (E2), paxillin (F2), and vinculin (G2) was shown by immunostaining with specific primary antibodies. The yellow signals in the merged images suggest colocalization of αPIX with calpain 4 (A3), calpain 1 (B3), calpain 2 (C3), ILK (D3), and β1 integrin (E3) in a dotted pattern at the plasma membrane (indicated by arrowheads). No colocalization was observed for HA-αPIX and paxillin (F3) or vinculin (G3). Selected regions are magnified to emphasize colocalization in small protein aggregates and to refer to their size of about 0.5–1.0 μm (A4–G4). The scale bars represent 10 μm (A1–G1) or 2.5 μm (A4–G4). The respective cells are shown by phase-contrast microscopy (A5–G5).
FIG. 5. Inhibition of m-calpain results in significant repression of integrin-mediated cell spreading that can be restored by overexpression of αPIX wild type and L386R/L387S, but not αPIX ΔDH. A, CHO-K1 cells were serum-starved for 24 h, trypsinized, and pretreated with culture medium containing either Me2SO, calpeptin, or calpain-inhibitor IV for 20 min. Subsequently, cells were replated onto fibronectin-coated coverslips and incubated for 30 min. Control cells were still able to spread, whereas cell spreading was significantly inhibited in cells treated with calpeptin or calpain inhibitor IV. Pictures were taken from representative areas out of three independent experiments. B, immunofluorescence analysis of CHO-K1 cells expressing various αPIX proteins in the presence of calpain inhibitor IV. Serum-starved CHO-K1 cells expressing HA-tagged αPIX wild type (B1), αPIX L386R/L387S (B3), or αPIX ΔDH (B5) were treated as described in A using calpain inhibitor IV. After a 30-min incubation, cells were fixed and permeabilized, and HA-αPIX was stained with anti-HA antibody. Cells expressing αPIX wild type or L386R/L387S extended lamellae protrusions in the presence of calpain inhibitor IV (arrowheads in B2 and B4), whereas those expressing αPIX ΔDH did not and appeared round (B6). Pictures were taken from representative areas out of three separate experiments. C, quantitative analysis of cell spreading in untransfected, αPIX wild type-, αPIX L386R/L387S-, or αPIX ΔDH-expressing CHO-K1 cells replated onto fibronectin in the presence of Me2SO, the calpain inhibitor calpeptin, or calpain inhibitor IV. The results shown are the means ± S.D. of three independent experiments. D, both αPIX wild type and αPIX L386R/L387S are able to bind calpain 4 in the presence of calpeptin or calpain inhibitor IV. The indicated HA-tagged αPIX constructs were cotransfected with GST-calpain 4 into CHO-K1 cells. Subsequently, cells were incubated in medium containing Me2SO, calpeptin, or calpain inhibitor IV for 14 h and lysed with buffer containing Me2SO, calpeptin, or calpain inhibitor IV. The GST-tagged protein complexes were isolated with glutathione-Sepharose beads and subjected to immunoblot analysis with anti-HA antibody. αPIX
of the actin cytoskeleton during the formation of cellular processes. Active calpain is necessary for lamellipodia formation, activation of Rho GTPases, and actin filament organization (25, 28). Calpain-cleaved β3 integrin was found in transient integrin clusters that form at early stages after integrin-induced adhesion of bovine aortic endothelial cells (29). Moreover, calpain cleaves RhoA during integrin-induced spreading, thereby generating a dominant negative form that significantly decreases cell spreading (42). Additional proteins have been detected in the early integrin clusters such as α-actinin and skelemin (41). Nonetheless, it is not known whether these molecules are cleaved by active calpain. Similarly, we cannot yet exclude the possibility that αPIX is cleaved by m-calpain in the early integrin clusters in CHO-K1 cells.

Inhibition of m-calpain in CHO-K1 cells led to significant impairment of cell spreading that could be restored by overexpression of αPIX wild type or the GEF activity-deficient L386R/L387S mutant, but not by the αPIX ΔDH mutant, suggesting that the interaction between αPIX and calpain 4/m-calpain is necessary for cell spreading rather than the GEF activity of αPIX. Indeed, association of calpain 4 and αPIX wild type or L386R/L387S mutant was found in the presence of calpain inhibitors. Together, these data suggest that αPIX acts downstream of calpain. Although the GEF activity of αPIX is not primarily necessary for restoring cell spreading in the presence of calpain inhibitors, the integrity of the αPIX DH domain seems to be required for this process. Besides cleaving integrin/cytoskeletal proteins, m- and μ-calpain are also involved in signal transduction pathways, e.g. they play a critical role during integrin-induced actin remodeling and cell spreading (27, 28). Inhibition of calpain with membrane-permeable inhibitors or by expression of a catalytically inactive form resulted in an inability of cells to spread. Remarkably, overexpression of constitutively active forms of Rac and RhoA reversed this effect, suggesting that calpain acts upstream of both Rac and RhoA in integrin-induced cell spreading in bovine aortic endothelial cells (28). It has been shown that μ-calpain but not m-calpain is involved in these morphological changes (28, 29).

In contrast, m-calpain but not μ-calpain was detected at sites of focal contact formation during T-cell adhesion and fibronectin-dependent spreading (43). We found m-calpain to be implicated in integrin-mediated cell spreading in CHO-K1 cells because inhibition of CHO-K1 cells with the m-calpain-specific calpain inhibitor IV abrogated integrin-dependent cell spreading. Conversely, inhibition of μ-calpain did not significantly disturb spreading. Because none of the synthetic calpain inhibitors presently available is completely specific for one calpain form, we cannot exclude an implication of μ-calpain in this process, for example at a different time point. In NIH 3T3 cells, stable overexpression of the natural calpain inhibitor calpastatin resulted in a decreased level of m-calpain mRNA and impaired the ability of cells to extend actin-rich processes such as lamellipodia and filopodia and to spread (27). In a recent study that aimed to address the isoform-specific functions of calpain 1 and calpain 2 in regulating membrane protrusion, it has been shown that calpain 2 (m-calpain) is necessary for the protrusion and lamellipodia formation at the leading edge (44). Together, these data implicate m-calpain in integrin-induced signaling events. It seems that both calpain isoforms are probably involved in integrin-mediated cell spreading and that the cell type and/or the stage of cell spreading determines which calpain form is required.

The association of αPIX with calpain 4 occurs via the SH3-DH-PH triple domain. In the majority of Rho GEF proteins, the DH-PH module is responsible for the exchange activity in vivo (45), whereas the SH3 domain is involved in interaction with...
other proteins, in case of αPIX with PAK (16, 17). The activity of GEF proteins is regulated by direct protein-protein interaction of the DH and/or PH domains with other molecules (45). For example, the catalytic DH-PH module of ephexin was shown to associate with the EphA receptor, suggesting that EphA modulation of ephexin activity might occur through hindrance of the GEF activity (46). A similar mechanism for modulation of αPIX GEF activity by calpain 4 might be possible.

Overexpression of wild-type αPIX dramatically enhanced the formation of cellular protrusions during integrin-dependent cell spreading, whereas no morphological changes were observed when the αPIX GEF-deficient L386R/L387S mutant or the ΔDH mutant was expressed. The protrusions formed upon overexpression of wild-type αPIX resemble lamellae sheets with long and thin filopodia-like structures at their end. αPIX is located in a punctual pattern at the end of these hand-like structures, which most likely represent early integrin clusters. Together, these data suggest that Rac and Cdc42 activity might be elevated in these cells. In the majority of reports, cell morphology changes rather than direct determination of Rac activity have been documented during integrin-mediated cell spreading at very early time points (11, 12, 28, 29, 41). Vav1 overexpression in Jurkat cells that were plated on fibronectin for 10 min did not induce an increase in Rac1 and Cdc42 activities, although the cells showed enhanced lamellipodia formation. Based on these findings, it has been suggested that integrin engagement induces activation of a very restricted Rac1/Cdc42 pool within the cell, resulting in a very modest increase in total Rac1/Cdc42 activity (47). Similarly, we were not able to detect increased Rac1 or Cdc42 activity by PBD (p21-binding domain of PAK) pull-down (data not shown). In line with this finding, the GEF activity of αPIX has been described to be very low (17, 40), and additional cofactors, e.g., PAK1, Cdc42, Rac1, platelet-derived growth factor receptor, Gβγ, and RP1 (calponin homology 1 domain of affixin), are required for the detection of αPIX exchange activity in vivo (18, 35, 39). However, αPIX exchange activity for Rac1 and Cdc42 has already been shown in vitro (17, 48) and seems to be differentially regulated depending on the monomer-dimer equilibrium of αPIX (49). Together, these observations suggest that although αPIX expression alone is not sufficient for detecting GEF activity in vivo, it is apparently adequate to cause morphological changes during cell spreading and in fully spread cells (32, 40, 47). Furthermore, our data suggest that the GEF activity of αPIX is required for enhanced formation of cellular protrusions, whereas a GEF activity-independent pathway might also exist. Exchange factor-independent functions of αPIX are known, e.g., PAK1 activity was enhanced solely by association with αPIX (39). Moreover, an exchange factor-independent role of the Rho, Rac, and Cdc42-specific GEF Vav1 (50) in integrin-mediated T-cell spreading has also been identified (47). Thus, we propose that αPIX may exert a dual effect: one depends on its GEF activity and leads to enhanced formation of characteristic cellular protrusions, and the other one regulates cell spreading via yet unidentified signaling components.

Recently, we identified an association of αPIX with β-parvin (affixin) (32), a novel focal adhesion protein (30, 31). β-Parvin binds to ILK (31), a serine/threonine protein kinase, which is able to associate with the cytoplasmic domains of β1 and β3 integrin (51) and binds to α-actinin in an ILK kinase activity-dependent manner (52). Both expression of the α-actinin binding domain of β-parvin in CHO-K1 cells and β-parvin knockdown by short interfering RNAs resulted in blockage of cell spreading and lamellipodia formation (52). These data are in contrast to those reported very recently by Zhang et al. (53) showing that depletion of β-parvin in HeLa cells replated on fibronectin for 25 min had no effect on cell spreading. The direct association of αPIX with β-parvin and calpain 4 as well as colocalization of αPIX with β1 integrin and ILK during cell spreading provides evidence that integrin-induced signaling leads to the formation of large protein complexes, the early integrin cluster (29). Thus, we suggest that upon attachment of cells to the extracellular matrix, early integrin clusters are formed containing β1 integrin, ILK, calpain proteases, β-parvin, α-actinin, and αPIX. These clusters promote cell spreading via at least two distinct mechanisms. Activation of Rho GTPases is mediated by the GEF exchange activity of αPIX and leads to reorganization of the actin cytoskeleton. However, an additional, αPIX GEF-independent signaling cascade exists that most likely depends on αPIX-calpain 4 association and results in cell spreading. In this pathway, calpain acts upstream of αPIX (Fig. 6).

Because mutations of αPIX (ARHGEF6) are implicated in X-linked nonspecific mental retardation in humans (54), the question arises whether integrin-dependent signaling and cell spreading might also be involved in the regulation of neurite outgrowth and the morphology of dendrites and/or dendritic spines. In this context, it is of interest to note that ILK was found to be important for integrin-dependent neurite outgrowth in N1E-115 cells (55) as well as nerve growth factor-mediated neurite outgrowth in rat adrenal phochromocytoma PC12 cells (56). However, additional studies are required to elucidate the biological function of αPIX in integrin-mediated signaling and cell spreading during neuronal processes.

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REFERENCES

1. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–518
2. Schoenwaelder, S. M., and Burridge, K. (1999) Curr. Opin. Cell Biol. 11, 274–286
3. Hotchin, N. A., and Hall, A. (1995) J. Cell Biol. 131, 1857–1865
4. van Aelst, L., and D’Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322
5. Hall, A. (1998) Science 283, 509–514
6. Ridley, A. J., and Hall, A. (1992) Cell 70, 389–399
7. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
8. Nobe, C. D., and Hall, A. (1995) Cell 81, 53–62
9. Burridge, K., and Wennerberg, R. (2004) Cell 116, 167–179
10. Barry, S. T., Flinn, H. M., Humphries, M. J., Critchley, D. R., and Ridley, A. J. (1997) Cell Adhes. Commun. 4, 387–398
11. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998) J. Cell Biol. 142, 573–586
12. Price, L. S., Leng, J., Schwartz, M. A., and Bokoch, G. M. (1998) Mol. Biol. Cell 9, 1863–1871
13. Ren, X. D., Kissoses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
14. Liu, B. P., and Burridge, K. (2000) Mol. Cell. Biol. 20, 7160–7169
15. Marignani, P. A., and Carpenter, C. L. (2000) J. Cell Biol. 154, 177–186
16. Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L., and Cerione, R. A. (1998) J. Biol. Chem. 273, 23633–23636
17. Manser, E., Lee, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 183–192
18. Yoshii, S., Tanaka, M., Otsuki, Y., Wang, D. Y., Guo, R. J., Zhu, Y., Takeda, R., Hanai, H., Kaneko, E., and Sugimura, H. (1999) Oncogene 18, 5680–5690
19. Bagrodia, S., and Cerione, R. A. (1999) Trends Cell Biol. 9, 350–355
20. Turner, C. E., Brown, M. C., Perrotta, J. A., Riedy, M. C., Nikopolouso, S. N., McDonald, A. R., Bagrodia, S., Thomas, S., and Leventhal, P. S. (1999) J. Cell Biol. 145, 851–863
21. Manabe Ri, R., Kovalenko, M., Webb, D. J., and Horwitz, A. R. (2002) J. Cell Sci. 115, 1497–1510
22. Premont, R. T., Perry, S. J., Schmalzigau, R., Roseman, J. T., Xing, Y., and Clang, A. (2004) Cell. Signal. 16, 1001–1011
23. West, A. R., Zhang, H., Brown, M. C., Nikopolouso, S. N., Riedy, M. C., Horwitz, A. F., and Turner, C. E. (2001) J. Cell Biol. 154, 161–176
24. Goll, D. E., Thompson, V. P., Li, H., Wei, W., and Cong, J. (2003) Physiol. Rev. 83, 731–891
25. Sato, K., and Kawashima, S. (2001) Biol. Chem. 382, 743–751
26. Glading, A., Lauffenburger, D. A., and Wells, A. (2002) Trends Cell Biol. 12, 46–54.
27. Potter, D. A., Tirnauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco, K. A., Mier, J. W., Maki, M., and Herman, I. M. (1996) J. Cell Biol. 141, 647–662
28. Kulkarni, S., Saido, T. C., Suzuki, K., and Fox, J. E. (1999) J. Biol. Chem. 274, 21265–21275
29. Bialkowska, K., Kulkarni, S., Du, X., Goll, D. E., Saido, T. C., and Fox, J. E. (2000) J. Cell Biol. 151, 685–696
30. Okita, T. M., Noegel, A. A., and Koenenbaum, E. (2001) J. Cell Biol. 141, 525–538
31. Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., and Ishigatsubo, Y. (2001) J. Cell Biol. 153, 1251–1264
32. Rosenberger, G., Jantke, I., Gal, A., and Kutsche, K. (2003) EMBO J. 22, 2324–2333
33. Brakebusch, C., and Fassler, R. (2003) Exp. Cell Res. 299, 179–187
34. Franco, S., Perrin, B., and Huttenlocher, A. (2004) Exp. Cell Res. 299, 179–187
35. Schmidt, A., and Hall, A. (2002) Genes Dev. 16, 1587–1609
36. Shamah, S. M., Lin, M. Z., Geldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A., and Greenberg, M. E. (2001) Cell 105, 233–244
37. del Pozo, M. A., Schwartz, M. A., Hu, J., Kiosses, W. B., Altman, A., and Villalba, M. (2003) J. Immunol. 170, 41–47
38. Feng, Q., Albeck, J. G., Cerione, R. A., and Yang, W. (2002) J. Biol. Chem. 277, 5644–5650
39. Feng, Q., Baird, D., and Cerione, R. A. (2004) EMBO J. 23, 3492–3504
40. Hornstein, I., Alcover, A., and Katzav, S. (2004) Cell. Signal. 16, 1–11
41. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolini, M. G., Radvka, G., Filsoos, J., Bell, J. C., and Dedhar, S. (1996) Nature 379, 91–96
42. Yamaji, S., Suzuki, A., Kanamori, H., Mishima, W., Yoshimi, R., Takakase, H., Takabayashi, M., Fujimaki, K., Fujisawa, S., Ohno, S., and Ishigatsubo, Y. (2004) J. Cell Biol. 165, 539–551
43. Zhang, Y., Chen, K., Tu, Y., and Wu, C. (2004) J. Biol. Chem. 279, 41695–41705
44. Kutsche, K., Yotema, H., Brandt, A., Janke, I., Novak, W. G., Orth, U., Baarova, M. G., David, D., Chelly, J., Fryns, J. P., Moraine, C., Rogers, H. H., Hamel, B. C., van Bokhoven, H., and Gal, A. (2000) Nat. Genet. 26, 247–250
45. Ishii, T., Satoh, E., and Nishimura, M. (2001) J. Biol. Chem. 276, 42994–43003
46. Mills, J., Dipicytioglu, M., Legg, A. T., Young, C. E., Young, S. S., Barr, A. M., Fletcher, L., O'Connor, T. P., and Dedhar, S. (2003) J. Neurosci. 23, 1638–1648