CLP-36 PDZ-LIM Protein Associates with Nonmuscle α-Actinin-1 and α-Actinin-4*

(Received for publication, January 10, 2000, and in revised form, January 20, 2000)

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The PDZ-LIM family of proteins (Enigma/LMP-1, ENH, ZASP/Cypher, RIL, ALP, and CLP-36) has been suggested to act as adapters that direct LIM-binding proteins to the cytoskeleton. Most interactions of PDZ-LIM proteins with the cytoskeleton have been identified in striated muscle, where several PDZ-LIM proteins are predominantly expressed. By contrast, CLP-36 mRNA is expressed in several nonmuscle tissues, and here we demonstrate high expression of CLP-36 in epithelial cells by in situ hybridization analysis. Our subcellular localization studies indicate that in nonmuscle cells, CLP-36 protein localizes to actin stress fibers. This localization is mediated via the PDZ domain of CLP-36 that associates with the spectrin-like repeats of α-actinin. Interestingly, immunoprecipitation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis indicate that both nonmuscle α-actinin-1 and α-actinin-4 form complexes with CLP-36. The high expression of α-actinin-4 in the colon, together with these results, suggests a specific function for the α-actinin-4–CLP-36 complex in the colonic epithelium. More generally, results presented here demonstrate that the association of PDZ-LIM proteins with the cytoskeleton extends to the actin stress fibers of nonmuscle cells.

PDZ and LIM domains are protein interaction motifs that are found in various proteins associated with the cytoskeleton (reviewed in Refs. 1 and 2). Recent characterization of several related proteins has revealed a new family of proteins that contain an N-terminal PDZ domain and one or more C-terminal LIM domains (3–6). ALP (5), RIL (7), and CLP-36 (8) each contain a single LIM domain, whereas Enigma/LMP-1 (10, 11), ENH (12), and ZASP/Cypher1 (4, 6) each have three C-terminal LIM domains. In addition, two more distantly related proteins, namely LIMK-1 (13) and LIMK-2 (14), contain a kinase domain C-terminal to a single PDZ and two LIM domains.

PDZ-LIM proteins have been suggested to act as adapters between kinases and the cytoskeleton (3, 6). This is based on two lines of studies indicating that PDZ-LIM proteins associate on one hand to the cytoskeleton via their PDZ domain (3–6), and on the other hand to kinases via their LIM domains (6, 11, 12, 15). The LIM-kinase interaction has been demonstrated with three PDZ-LIM proteins, mostly using yeast two-hybrid screens. Enigma was identified by virtue of association of its third LIM domain to the cytoplasmic tail of the insulin receptor; this interaction is apparently important for endocytosis of the receptor (11). The association of the second LIM domain of Enigma with Ret/ptc2 is required for the plasma membrane localization and mitogenic activity of Ret/ptc2 (15, 16). ENH was discovered through its binding to protein kinase C (12), and subsequently, Cypher1 was also found to associate with protein kinase C (6). The association of the LIM domain of RIL with the second and fourth PDZ domains of protein tyrosine phosphatase PTP-BL in a yeast two-hybrid screen (17) suggests that the LIM domain interactions of PDZ-LIM proteins may not be limited to kinases.

The association of PDZ domains with the cytoskeleton in PDZ-LIM proteins has been mostly studied in muscle due to their high specific expression in this tissue (3–6). Enigma, ALP, and ZASP/Cypher1 proteins localize to the Z line of striated muscle. This localization is mediated by the association of Enigma PDZ domain with β-tropomyosin (3) or by the association of ALP and ZASP/Cypher1 PDZ domains with α-actinin-2 (4–6).

α-Actinin-2 and α-actinin-3 are the muscle-specific α-actinins forming part of the contractile machinery anchoring actin thin filaments at the Z lines and dense bodies in striated and smooth muscle, respectively (18). Cellular α-actinin exists as an antiparallel dimer with a globular head domain, spectrin-like repeats and EF-hands (reviewed in Ref. 19). Dimerization of α-actinin is mediated by the spectrin-like repeats of α-actinin (20–22). This rod domain mediates the association with the PDZ domain of ALP, whereas ZASP binds to a 155-amino acid C-terminal region of α-actinin-2 (5, 6).

In addition to the muscle-specific α-actinins, two nonmuscle human α-actinin isoforms, α-actinin-1 (23) and α-actinin-4 (24), have been identified and represent independent genes. α-Actinin-1 is localized along stress fibers and takes part in bundling actin filaments (25). It also associates with several cytoskeletal and membrane associated proteins, such as integrins (26), intercellular adhesion molecules (27), N-methyl-D-aspartate receptor (28), and vinculin (29).

The subcellular localization of nonmuscle α-actinin-4 differs from that of α-actinin-1 in that α-actinin-4 is less clearly concentrated to stress fibers and is not detected in focal adhesions or cell contacts (24). Moreover, localization is cell type-dependent, sometimes demonstrating nuclear staining (24). Unlike α-actinin-1, the expression of α-actinin-4 is induced in migrating cells (24).

During studies on proteins associated with a partially characterized novel kinase,1 we became interested in the CLP-36 PDZ-LIM protein. CLP-36 (also called hCLIM1; Ref. 9) was initially identified as a rat gene down-regulated during hypoxia in hepatocytes (8). CLP-36 mRNA is expressed in several non-

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* This study was supported by grants from the Academy of Finland, the Finnish Cancer Society, the Finnish Cancer Institute, and the Sigrid Juselius Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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muscle tissues (8, 9), and here we demonstrate high expression of CLP-36 in epithelial cells by in situ hybridization analysis. Moreover, CLP-36 was found to localize to actin stress fibers via its PDZ domain through its association with cellular α-actinin-1 and α-actinin-4, suggesting that CLP-36 acts as an adapter between stress fibers and LIM-binding proteins in nonmuscle cells.

**EXPERIMENTAL PROCEDURES**

**In Situ Hybridization**—Embryos of CBA × NMRI mice at stages E7, E9, E11, E15, and E17.5 were timed by both vaginal plugs of mothers and by morphological criteria. The experiments were approved by the Animal Welfare Committee of the Haartman Institute, University of Helsinki. Tissue preparation and in situ hybridization using a antisense or sense RNA probe generated from human CLP-36 cDNA (nucleotides 87–1504 in GenBank™ accession number U90878) were performed as described (30).

**Mammalian Expression Vectors and Transfections**—The Myc-tagged CLP-36 plasmid was generated by subcloning an EcoRI-Xhol fragment from 38/JG4–5 (nucleotides 87–1504 in GenBank™ accession number U90878) into EcoRI-SolI sites of pAMC (31). Myc-tagged CLP-36 was done similarly using 51/JG4–5 (nucleotides 212–1504 in GenBank™ accession number U90878). U2OS osteosarcoma or COS-7 cells were transfected by using the calcium phosphate transfection method as described (32).

**Antibodies**—Mouse monoclonal (9E10) or rabbit polyclonal (PRB-150C) antibodies against Myc epitopes were from Babco Inc. (Berkeley, CA). The following α-actinin antibodies were used: A5044 mouse monoclonal antibody to detect α-actinin-1 (Sigma), NCC-Lu-632 mouse monoclonal antibody to detect α-actinin-4 (a kind gift of Dr. Hirohashi; Ref. 24), and a rabbit polyclonal α-actinin antibody (33).

**Immunofluorescence, Immunoprecipitation, and Western Blotting**—Transfected cells on coverslips were fixed with 3.5% (v/v) paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, labeled with antibodies or Hoechst, and analyzed as described (31). The 0.5% Triton X-100 extraction or the 1-h cytochalasin B treatment prior to fixation were performed as described (34). Immunoprecipitations were performed as described (31) except that the cleared supernatants in ELB (150 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2.5 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol-phosphate, 1 μg/ml leupeptin) were incubated with 1 μl of monoclonal anti-Myc antibody for 2 h at +4 °C prior to 1 h immunoprecipitation. Western blotting analysis was according to standard procedures (32).

**Generation of Recombinant Proteins and Solution Binding Assays**—For expression of GST-CLP-36, a NorI fragment from 38/pAMC was subcloned into pAcGHLT-A (Pharmingen, San Diego, CA) baculovirus transfer vector, which was introduced into Sf9 insect cells together with the CLP-36 plasmid was generated by subcloning an M36, 200 μg of COS-7 cell extracts in ELB were incubated with the indicated amounts of GST-CLP-36 for 2 h at +4 °C prior to adding 7.5 μl (packed volume) of glutathione-Sepharose beads for 1 h. Subsequently, the beads were washed four times with ELB and subjected to SDS-PAGE and Coomassie staining (see Fig. 6). For mapping of the CLP-36-α-actinin association, 200 μg of transfected COS-7 cell lysate in ELB were incubated with 4 μg of GST-α-actinins for 2 h at +4 °C prior to anti-Myc immunoprecipitation, SDS-PAGE, and Western blotting analysis (see Fig. 4B).

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a BiFlex™ time-of-flight instrument equipped with a nitrogen laser operating at 337 nm. CLP-36 associated α-actinin band from either U2OS or COS7 cells was cut out from the Coomassie-stained gel, digested with trypsin, eluted, and analyzed in the linear positive ion delayed extraction mode. The output was analyzed using ProFound.

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**RESULTS**

**High Expression of CLP-36 in Embryonal and Adult Epithelial Cells**—Northern blot analyses of CLP-36 indicated expression in various tissues with some variability between rat (8) and human (9) tissues. To determine the pattern of expression of CLP-36 in adult tissues and during embryogenesis, in situ hybridization analysis with an antisense CLP-36 mRNA probe and counterstained with hematoxylin of oral cavity epithelium (OC) and upper first molar tooth germ from a E17.5 stage embryo demonstrating prominent CLP-36 expression in basal epithelial cells in contrast to low expression in differentiating dental epithelium (DE). D and E, bright-field (D) and dark-field (E) images of adult small intestine epithelium (IE) and surrounding smooth muscle (SM).

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*The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.*
Association of CLP-36 with Nonmuscle α-Actinin

**CLP-36 Is Localized to Actin Stress Fibers**—The expression of CLP-36 in epithelium and other nonmuscle tissues prompted us to study its subcellular localization in cell lines of nonmuscle origin. To this end, a plasmid expressing a Myc epitope-tagged CLP-36 (Myc-CLP-36) or vector control was transiently transfected into U2OS osteosarcoma (Fig. 2) or COS-7 green monkey kidney epithelial cells (not shown). Double fluorescence analysis with anti-Myc and rhodamine-labeled phalloidin from vector transfected or Myc-CLP-36 transfected cells indicated partial colocalization of CLP-36 with filaments resembling actin stress fibers (Fig. 2A). To verify this colocalization, similar coverslips were subjected to detergent extraction or treated with cytochalasin B prior to fixation. As demonstrated in Fig. 2B (Triton X-100), detergent extraction revealed colocalization of CLP-36 and actin stress fibers. If microfilament formation was inhibited by cytochalasin B prior to fixation, CLP-36 was partially accumulated with the disrupted actin-containing filaments (Fig. 2B, cytochalasin B). These experiments indicate that a significant fraction of CLP-36 is associated with actin stress fibers.

**CLP-36 Associates with Cellular α-Actinin via Its PDZ Domain**—During the immunoprecipitation studies, it was noted that whenever Myc-CLP-36 was purified from cell lysates, a prominent associated protein of approximately 100 kDa was detected when staining for total protein. The size of the polypeptide together with the immunofluorescence pattern of CLP-36 suggested that the band represented α-actinin. Moreover, based on interactions of the other PDZ-LIM proteins (3–6), the association was further predicted to be mediated by the PDZ domain of CLP-36. To test this hypothesis, immunoprecipitates from cells expressing either Myc-CLP-36 or a mutant lacking part of the PDZ domain (Myc-CLP-Δ1–24) were analyzed by Western blotting with an α-actinin antibody (33). α-Actinin was only detected in the lane immunoprecipitated with the full-length CLP-36 (Fig. 3A), indicating that the first 24 amino acids of CLP-36 are required for α-actinin binding and thus suggesting that an intact PDZ domain is required for binding. The inability of Myc-CLP-Δ1–24 to associate with α-actinin was associated with loss of localization to stress fibers (Fig. 3B). This indicates that CLP-36 localization to stress fibers is mediated through its association with α-actinin.

The Spectrin-like Repeats of α-Actinin Interact with CLP-36—Previously PDZ-LIM proteins have been reported to associate with two distinct regions of α-actinin (4, 5). Therefore, the interaction site of α-actinin with CLP-36 was mapped using three fragments of α-actinin fused to GST. GST-ABD/R1-R2 contains the actin-binding domain and spectrin-like repeats 1 and 2; GST-R1-R4 contains all four spectrin-like repeats; GST-R3-R4/EF contains spectrin-like repeats 3 and 4 and the EF-hand. Bacterially produced proteins (Fig. 4A) were incubated with Myc-CLP-36 expressing or control cell extracts. Following anti-Myc immunoprecipitation and SDS-PAGE, the presence of
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Myc-CLP-36 (Fig. 4B, Myc) and of copurified \textit{α}-actinin fragments was analyzed (Fig. 4B, \textit{α}-actinin). The results indicated the presence of significant amounts of GST-R1-R4 in CLP-36 immunoprecipitates. Low levels GST-ABD/R1-R2 were also detected specifically in CLP-36 immunoprecipitates. The presence of GST-R3-R4/EF was due to unspecific binding, as it was also detected in the control lane.

Association of the endogenous \textit{α}-actinin with CLP-36 was also noted in all cases, as expected (Fig. 4B, \textit{α}-actinin). However, the level of associated \textit{α}-actinin decreased in the presence of GST-R1-R4 indicating competitive binding of these with CLP-36. As endogenous \textit{α}-actinin and GST-R1-R4 were detected using the same antibody, the results also demonstrated that the total amount of CLP-36-associated GST-R1-R4 was significantly higher than CLP-36-associated endogenous \textit{α}-actinin. This indicates that association of GST-R1-R4 with CLP-36 is not mediated indirectly through dimerization with endogenous \textit{α}-actinin. As the levels of CLP-36-associated GST-ABD/R1-R2 were lower than those of endogenous \textit{α}-actinin, the same conclusion cannot be made for GST-ABD/R1-R2. Taken together, these results indicate that the efficient association of CLP-36 with \textit{α}-actinin is mediated via the spectrin-like repeats.

\textbf{Cellular \textit{α}-Actinin-1 and \textit{α}-Actinin-4 Associate with CLP-36—}The expression of CLP-36 in nonmuscle cells suggested that it could associate with either \textit{α}-actinin-1 or the closely related \textit{α}-actinin-4. To investigate this, double fluorescence analysis of U2OS cells expressing Myc-CLP-36 was performed with anti-Myc and either A5044 to detect \textit{α}-actinin-1 or NCC-Lu-632 to detect \textit{α}-actinin-4. As shown in Fig. 5A, Myc-CLP-36 was colocalized both with \textit{α}-actinin-1 and \textit{α}-actinin-4 specifically in stress fibers and was more clearly detected after Triton X-100 pretreatment. CLP-36 was not detected in areas of focal adhesions. In addition, anti-Myc immunoprecipitates from Myc-CLP-36 expressing cells contained both \textit{α}-actinin-1 and \textit{α}-actinin-4 (Fig. 5B).

To estimate the stoichiometry of binding of \textit{α}-actinin to CLP-36, varying amounts of the GST-CLP-36 protein were incubated with 200 \textmu g of COS-7 cell lysate and subsequently purified using glutathione-Sepharose beads and analyzed with a Coomassie stain of SDS-PAGE (Fig. 6). A prominent band of approximately 100 kDa representing \textit{α}-actinin was the only band detected in addition to GST-CLP-36. An approximate mass ratio of 2:3 for GST-CLP-36 and \textit{α}-actinin was linear until \textit{α}-actinin in the lysate became limiting. Considering the molecular masses of GST-CLP-36 (66 kDa) and \textit{α}-actinin (103 kDa) this mass ratio indicates an equimolar complex, and together with the higher affinity of CLP-36 to actinin dimers, it suggests that two molecules of CLP-36 are associated with an \textit{α}-actinin dimer. The efficient purification of soluble \textit{α}-actinin from the cell extracts using GST-CLP-36 also enabled analysis of this protein by matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis, which indicated the presence of both \textit{α}-actinin-1 and \textit{α}-actinin-4 in the 100-kDa band.

\textbf{DISCUSSION—}The present study demonstrates that nonmuscle \textit{α}-actinin-1 and \textit{α}-actinin-4 associate with the PDZ domain of CLP-36 in actin stress fibers. This, together with the abundant expression of CLP-36 in skin and intestinal epithelia and other nonmuscle tissues, implicates a role for it as an adapter between stress fibers and LIM-binding proteins in these tissues. The high expression of \textit{α}-actinin-4 in the colon and in epithelial cells (24) suggests a specific function for the \textit{α}-actinin-4-CLP-36 complex.
CLP-36 and associated proteins were purified and subjected to SDS-PAGE and Coomassie staining. α-Actinin was the only protein that was detected associating with CLP-36 in stoichiometric amounts indicating direct association. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of this band indicated that it contained both α-actinin-1 and α-actinin-4.

in the colonic epithelium.

The expression pattern observed in the mouse in situ hybridization analyses is in concordance with previous Northern blotting data from rat tissues (8), both indicating that CLP-36 is not expressed in skeletal muscle. However, a positive signal in Northern blotting analysis from human skeletal muscle (9) suggests either species-specific expression differences or the presence of a closely related human gene. Analysis of expressed sequence tag data base content did not reveal closely related cDNAs, and 8 of 244 (3.3%) human CLP-36 expressed sequence tags were from skeletal muscle, supporting low relative expression of human CLP-36 in this tissue.

Previous studies have implicated roles for PDZ-LIM proteins in skeletal muscle. This is based both on the muscle-oriented laboratory (especially Päivi Ojala) is acknowledged for fruitful discussions.

## Acknowledgments
We thank Nisse Kalkkinen for mass spectrometry analysis; Birgitta Tjäder for technical assistance; and Drs. O. Carpen, S. Hirohashi, and D. Critchley for reagents. The Mäkelä laboratory (especially Päivi Ojala) is acknowledged for fruitful discussions.

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