The PDZ-LIM Protein CLP36 is required for stress fiber formation and focal adhesion assembly in BeWo cells

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Abstract

CLP36 belongs to the ALP subfamily of PDZ-LIM proteins and has a PDZ domain at its N-terminal and a LIM domain at its C-terminal. It has been shown that CLP36 is localized to stress fibers through interaction with alpha-actinin, but its function is still unclear. To investigate the role of CLP36 in stress fibers, we suppressed CLP36 expression in BeWo cells by RNAi and examined the phenotypic changes. CLP36-knockdown resulted in cell spreading and the loss of stress fibers and focal adhesions. These changes were reversed by addition of exogenous CLP36, but not by addition of mutant forms of CLP36 that lacked the PDZ or LIM domain. These findings indicate that CLP36 plays a critical role in stress fiber formation and the assembly of focal adhesions in BeWo cells. In addition, the PDZ and LIM domains are both essential for CLP36 to function.

Introduction

The actin cytoskeleton of a eukaryotic cell plays a critical role in cell motility, migration, and the maintenance of cell shape and polarity. Organization and function of the actin cytoskeleton are regulated by a variety of actin-binding proteins [1], among which alpha-actinin is a ubiquitous protein found in numerous actin structures. Apart from its interaction with actin, it has become clear that alpha-actinin is capable of binding to many other cytoskeletal or regulatory proteins [2]. It is therefore thought that alpha-actinin does not only act as a cross-linker of actin filaments, but also serves as a platform for the assembly of multiprotein complexes.

There is a family of PDZ-LIM proteins that each possess an N-terminal LIM domain and from one to three C-terminal LIM domains. Among them, CLP36 [3-6], mystique [7,8], ALP [9], and RIL [10] have a single LIM domain at the C-terminal, while ENH [11], Enigma [12], and Cypher1/ZASP [13,14] have three LIM domains at the C-terminal. It has been shown that a group of PDZ-LIM proteins, which comprises Cypher1/ZASP, ENH, CLP36, mystique, ALP, and RIL, associates with the actin cytoskeleton through interaction with alpha-actinin. In addition, ENH and Cypher1/ZASP have the ability to interact with PKC via its LIM domain [14,15], and CLP36 has been shown to bind with Clik1 kinase via its LIM domain [16]. Based on these findings, the PDZ-LIM proteins are hypothesized to act as adapter molecules that recruit signaling molecules to the actin cytoskeleton.

Cypher1/ZASP and ALP are muscle-specific proteins that are localized to the Z-discs of striated muscles. ALP-deficient mice develop right ventricular dilation and dysfunction [17], while Cypher1-deficient mice die of severe congenital myopathy [18]. These findings indicate that Cypher1/ZASP and ALP have a role in normal sarcomere function. On the other hand, CLP36, mystique, and RIL are predominantly expressed in other tissues rather than in the muscles. Strong expression of CLP36 has been observed in the heart, liver, lung, and endothelium of the esophagus and small intestine [5,6]. Previous studies have shown that CLP36 is localized to stress fibers via due to binding with non-muscular forms of alpha-actinin in non-muscle cells [3,4]. However, the function of CLP36 has not yet been elucidated.

Stress fibers are sarcomere-like contractile structures that are composed of actin filaments, alpha-actinin, myosin II, and other actin-binding proteins. It has been

established that the formation of stress fibers is regulated by a signaling cascade that involves RhoA small GTPase [19], but the mechanism by which these actomyosin contractile structures are generated is not well understood. The present study was designed to examine whether CLP36 had a role in the mechanism of stress fiber formation. We employed a trophoblast-derived choriocarcinoma cell line (BeWo) to generate CLP36-knockdown cells. We found that BeWo cells failed to develop stress fibers and focal adhesions in the absence of CLP36.

Materials and methods

Tissue collection

Wister male rats (200 g) were deeply anesthetized with diethylether, sacrificed, and tissue samples were collected. Placentae were collected from Wister rats (18 days pregnant) in the same way. These animals were purchased from SLC (Shizuoka, Japan). The procedures described were performed according to Animal Ethical Guidelines and approved by Hamamatsu University School of Medicine.

Cells and transfection

BeWo cells (JCRB9111; provided by HSRRB, Japan) were maintained in HAM's F-12 medium supplemented with 15% fetal bovine serum and antibiotics. Transient transfections were done using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions. For immunofluorescence staining, cells were reseeded on fibronectin (10 μg/ml)-coated coverglasses 24 h after transfection and cultured for additional 48 h.

Expression plasmids

Rat CLP36 cDNA (amino acids 2-327) was amplified by PCR using Rat Brain Marathon-Ready cDNA library (Clontech) as a template, and amplified DNA fragments were subcloned in frame into the EcoRI site of the pEGFP-C2 vector. CLP36 ΔPDZ (amino acids 80-327) or CLP36 ΔLIM (amino acids 2-262) cDNAs were obtained by PCR using the pEGFP-CLP36 plasmid as a template and ligated into the EcoRI site of the pEGFP-C2 vector. Nucleotide sequences of all these plasmids were verified by DNA sequencing.

GST-fusion proteins

Rat CLP36 cDNA was excised from the pEGFP-CLP36 plasmid and ligated into the EcoRI site of the pGEX-5X-1 vector (Amersham). The recombinant plasmid as well as insertless pGEX-5X-1 was transfected into *Escherichia coli* B21 and recombinant proteins were induced according to the manufacture's instructions.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in PBS for 15 min, blocked in PBS containing 0.3% (v/v) Triton X-100, 1% (v/v) goat serum and 1% (w/v) bovine albumin for 15 min, and incubated with anti-CLP36 antibody (1:200; BD Biosciences) or anti-alpha-actinin antibody (BM-75.2 1:200; Sigma) for 3h. After washing, they were incubated with Alexa-Fluor 488- or 594-conjugated secondary antibodies (1:1000; Molecular Probes). To detect filamentous actin, sections were incubated with Alexa-Fluor 594-conjugated phalloidin (1:200; Molecular Probes). For visualization of nuclear location, cells were covered with mounting medium containing 4', 6-diamidino-2-phenylindoleindoleincubated (DAPI) (Vector lab).

RNA interference experiments

RNA targeting constructs were generated using pSUPERIOR. neo+gfp vector (OligoEngine) according to the manufacture's instructions. Nineteen nucleotide sequences to target human CLP36 were determined by SiDirect web site. The sequences used in this study were as follows: (A) 5'-GCAAGGCGGCTCTAGCTAA-3', (B) 5'-GCAGCCTTGTCATCGACAA-3', and (C) 5'-GTGGCTGCGTCGATTGGAA-3'. BeWo cells were transfected with these pSUPERIOR constructs and selected by a two-week incubation with 500 μg/ml G418 (Sigma).

Western-blot analysis, immunoprecipitation

Tissue samples or cells were homogenized in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1.5 mM $MgCl_2$, 4 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and complete protease inhibitor cocktail (Roche Diagnostics)]. After centrifugation at 15,000 rpm for 30 min, the supernatant was taken as a loading sample. Protein samples (15 μg each) were separated by SDS–PAGE and transferred to a

nitrocellulose membrane (Schleicher & Schuell). After blocking with 5% (w/v) non-lat dry milk in PBS for 60 min, the membranes were incubated with first antibodies overnight at 4°C. After washing, bound Igs were visualized with secondary antibodies conjugated to horseradish peroxidase (1:5,000; Nacalai Tesque). Protein bands were detected using ECL detection system (Pierce). For an absorption test, the CLP36 antibody was incubated with 20 μg of GST-CLP36 or GST alone overnight prior to the immunoreaction. Dilutions of first antibodies were as follows: anti-CLP36 (1:500), anti-alpha-tubulin (1:1000; Sigma), anti-FAK (1:1000; BD Biosciences), anti-paxillin (1:1000; BD Biosciences), anti-alpha-actinin (1:1000; Sigma), anti-myosin II (1:1000; Sigma), anti-beta-actin (1:1000; Sigma), and anti-vinculin (1:1000, Sigma). For immunoprecipitation, cells were homogenized in lysis buffer [25 mM Tris, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% (v/v) Triton X-100 and complete protease inhibitor cocktail]. After centrifugation, the supernatant was incubated with Protein G Sepharose bound with anti-CLP36 antibody or control mouse IgG for 2 h and proteins retained on the beads were eluted in SDS-PAGE sample buffer.

Morphological Analysis.

Cells were plated on fibronectin (10 μg/ml)-coated glass base dishes (Iwaki) and cultured for 48 h under appropriate conditions. After fixation with 4% paraformaldehyde, cells were subjected to morphological analyses. Phase-contrast images were taken with a BZ-8000, and measurement of the distance and calculation of the area were done using a BZ-analyzer. In each experiment, approximately 100 cell images were captured and analyzed. Statistical analysis was performed using Statview 4.5 software.

Results

Initially, we investigated the tissue distribution of CLP36 in rats and found a high content of CLP36 in the rat placenta, heart, lungs, liver, and spleen (Fig. 1A). This led us to consider that a trophoblast-derived choriocarcinoma cell line (BeWo) might be a good model for investigating the cellular functions of CLP36. Western-blot analysis showed that BeWo cells express CLP36 (Fig. 1B). When cultured on fibronectin-coated coverslips, BeWo cells developed stress fibers, and CLP36 and alpha-actinin were both

localized to these fibers (Fig. 1C). Co-immunoprecipitation experiments demonstrated that endogenous CLP36 formed a complex with alpha-actinin in BeWo cells (Fig. 1D). These findings were similar to those obtained in other cell lines examined previously [3-5]. Therefore, we concluded that this cell line was an appropriate model for studying the function of CLP36.

To investigate the role of CLP36 in stress fibers, we generated CLP36-deficient BeWo cells using short hairpin (sh) RNA plasmids and assessed the effect of CLP36-knockdown. As shown in Fig. 2A, three kinds of CLP36-targeting shRNAs efficiently reduced the expression of endogenous CLP36 protein, whereas the expression of other stress fiber components (such as alpha-actinin, myosin II, and beta-actin) was not affected. On the other hand, mock transfection did not alter the expression of CLP36 or the other proteins. Phase contrast microscopy revealed several prominent morphological changes in CLP36-knockdown cells. These cells developed a ruffled membrane and displayed a round shape (Fig. 2, B and C), which was in contrast to the fibroblast-like features of wild-type cells. Development of membrane extensions led to a flattered morphology and CLP36-knockdown cells had an area about 1.5 times that of wild-type cells (Fig. 2D). In contrast, mock transfection had no effect on the morphological characteristics of BeWo cells (not shown).

Observation by phase-contrast microscopy suggested that depletion of CLP36 altered the organization of the actin cytoskeleton. Indeed, phalloidin staining revealed that stress fibers had been lost from CLP36-knockdown cells (Fig. 3, Aa and B), while actin filaments and alpha-actinin were localized to the cell periphery (Fig. 3A). Furthermore, vinculin staining showed dot-like focal complexes at the cell periphery, which were in contrast to the dash-like focal adhesions seen in wild-type cells (Fig. 3C). The expression of focal adhesion components (vinculin, paxillin, and FAK) was unaffected by CLP36-knockdown (Fig. 3D). Therefore, these findings indicate that CLP36 is essential for the assembly of stress fibers as well as for the maturation of focal complexes into focal adhesions.

To establish that the alterations described above were directly caused by CLP36-knockdown, we next tested whether they could be reversed by addition of exogenous CLP36. At 72 h after transfection of GFP-CLP36, stress fibers were restored in about 40% of the transfected cells, while GFP-CLP36 and alpha-actinin were co-localized on the stress fibers (Fig. 4B and C). Following the development of stress

fibers, the transfected cells tended to revert to a fibroblast-like morphology. In addition, alpha-actinin staining showed that dash-like focal adhesions formed at the ends of the stress fibers (Fig. 4B, arrows). In contrast, cells transfected with GFP alone were devoid of stress fibers and focal adhesions (Fig. 4A and B).

CLP36 possesses PDZ and LIM domains. We next investigated whether these domains are necessary for stress fiber formation. To address this issue, we constructed a CLP36 mutant lacking the PDZ domain (ΔPDZ) and one lacking the LIM domain (ΔLIM), and then transfected CLP36-knockdown cells with these mutants. Although both mutants were able to interact with alpha-actinin, stress fibers failed to develop (Fig. 4). Instead, amorphous actin filament networks were observed in the cytoplasm of cells expressing these mutants. We observed that transfection with GFP-CLP36 ΔLIM reduced the concentration of alpha-actinin at the cell periphery. However, GFP-CLP36 ΔPDZ did not alter the co-localization of alpha-actinin at the cell periphery. These findings suggested that the PDZ and LIM domains both play essential, but different, roles in the actions of CLP36.

Discussion

This is the first report about the cellular functions of CLP36. We found that CLP36 was localized to stress fibers in BeWo cells. Its depletion brought about alterations of cell morphology, and both stress fibers and focal adhesions were lost in the knockdown cells. Addition of exogenous CLP36 restored a normal morphology the knockdown cells, along with the return of stress fibers and focal adhesions. Depletion of CLP36 did not affect the expression levels of other proteins constituting the stress fibers and focal adhesions (as far as we tested). These findings indicate that CLP36 is essential for the assembly of stress fibers and focal adhesions.

Instead of focal adhesions, CLP36-knockdown cells generated dot-like focal complexes at the leading edge of their membrane ruffles. It has been shown that the assembly and maintenance of focal adhesions depends on the tension generated by stress fibers [20]. In response to increased contractility of actomyosin, focal complexes grow and mature into focal adhesions [21]. Conversely, a decrease of tension due to myosin phosphorylation or myosin II knockdown causes the loss of focal adhesions and stress fibers [22,23]. Bauer et al. demonstrated that CLP36 is localized to stress fibers,

but absent from focal adhesions, in endothelial cells, suggesting that CLP36 is not directly involved in the organization of adhesions [3]. Taken together, these findings suggest that reduction of cell-generated tension caused by loss of stress fibers impairs the maturation of focal complexes into focal adhesions in CLP36-knockdown cells.

There are several possible explanations for the loss of stress fibers after knockdown of CLP36. It has been suggested that PDZ-LIM proteins act as adapters that link signaling molecules to the actin cytoskeleton. The LIM domains of PDZ-LIM proteins have been shown to interact with several kinases. Vallenius and Mäkelä reported that CLP36 binds to Clik1 kinase via its LIM domain and that CLP36 has the ability to recruit nuclear Clik1 kinase to stress fibers [16]. It is known that stress fiber formation is regulated by a signaling cascade which involves small GTPase RhoA [19]. Although it is unclear if Clik1 is a regulator of actin stress fibers, CLP36 may be one of the important adapter molecules involved in the RhoA signaling cascade.

Another possibility is that CLP36 may be required for normal functioning of alpha-actinin. CLP36 has two binding sites for alpha-actinin, which are located at the PDZ and midway between the PDZ and LIM domains [3,4]. It has been suggested that the conformation of alpha-actinin may be stabilized if its two binding sites are occupied by a single molecule of CLP36 [24], so binding with CLP36 may be necessary for alpha-actinin to assume a suitable conformation for cross-linking. Alternatively, cross-linking of alpha-actinin may be regulated by Clik1 or another kinase that binds to the LIM domain of CLP36. Further investigation will be required to define the precise role of CLP36 in stress fiber formation.

The occurrence of stress fibers has been documented not only in cultured cells but also in endothelial cells of the vascular system. In particular, stress fibers are well developed at sites exposed to high levels of shear stress due to blood flow, i.e., the endothelial cells of arteries, cells in the splenic sinuses [25,26]. As shown in Fig. 1A, CLP36 is highly expressed in the heart, lungs, liver, and spleen as well as the placenta. Thus, it seems possible that CLP36 is expressed by the vascular endothelial cells and plays a role in stress fiber formation *in vivo*. To verify this hypothesis, immunohistochemical studies will need to be done in the future.

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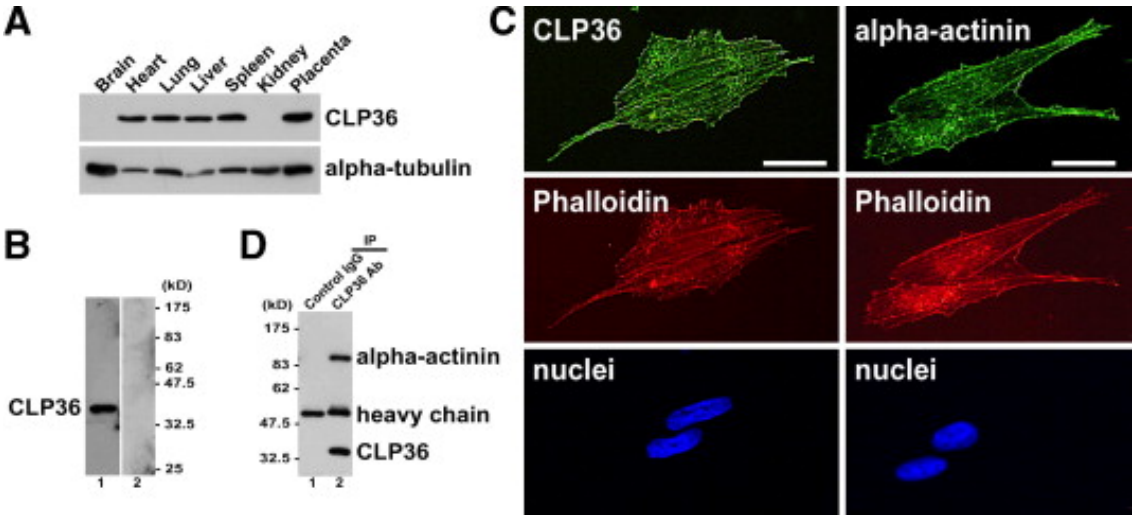


Fig. 1. Co-localization of CLP36 and alpha-actinin at stress fibers in BeWo cells. (A) Western-blot analysis showing the tissue distribution of CLP36 in the rat. Protein samples (15 µg) from indicated tissues were loaded per lane and probed with anti-CLP36 antibody. As a control, the membrane was reprobed with anti-alpha-tubulin antibody. (B) Anti-CLP36 antibody recognized endogenous CLP36 proteins in BeWo cells (lane 1). No reaction with the antibody preadsorbed on GST-CLP36 (lane 2). (C) Double staining of BeWo cells with anti-CLP36 or anti-alpha-actinin antibody and phalloidin. Nuclear localization was visualized with DAPI. Bars, 50 µm. (D) Co-immunoprecipitation. BeWo cell lysates were immunoprecipitated with anti-CLP36 antibody (lane 2) or control IgG (lane 1), and analyzed for anti-alpha-actinin and anti-CLP36 antibodies by Western-blot.

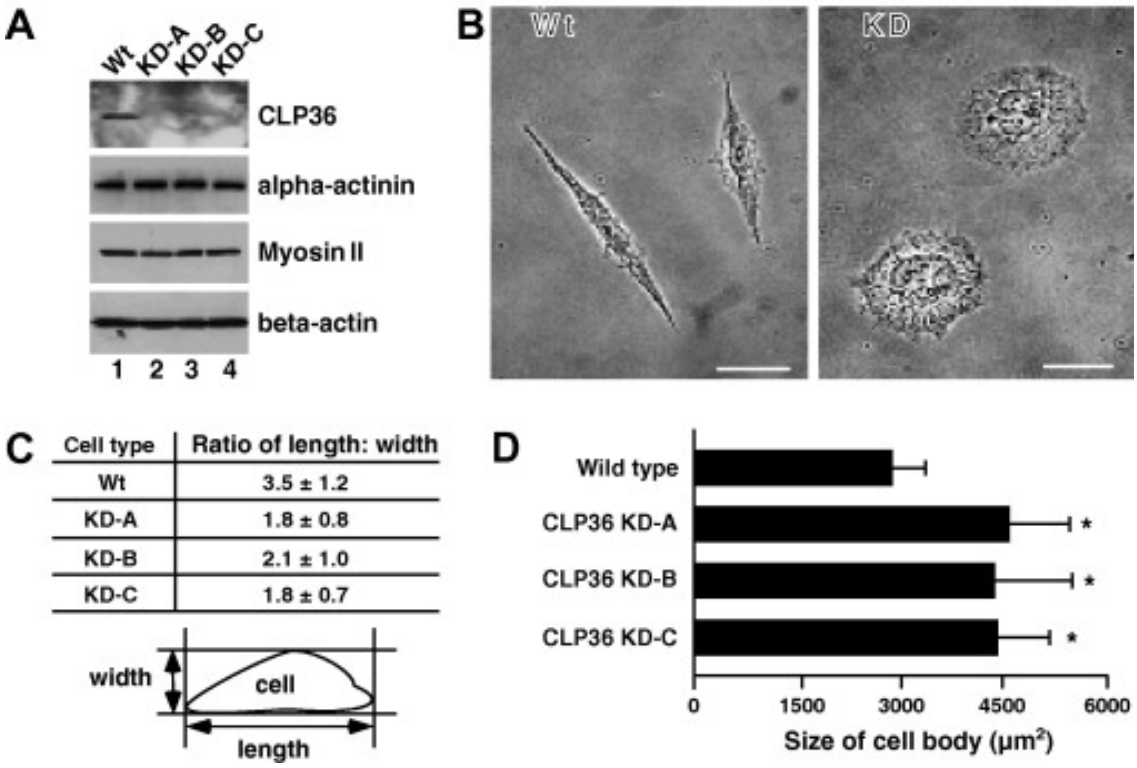


Fig. 2. Effects of CLP36-knockdown on cell morphology. (A) Three lines of CLP36-knockdown BeWo cells were generated by RNAi with different target sequences. Western-blot analysis showing that CLP36 expression was specifically suppressed in the three knockdown cell lines. (B) Phase-contrast images of wild-type BeWo cells and CLP36-knockdown cells (cell line C). CLP36-knockdown cells developed membrane ruffles in all directions and became a round shape. Bars, 50 μm. (C) Alternations of cell shape after CLP36-knockdown were quantified by the ration of the length/width. Values are means ± standard error for 75 cells. (D) Histograms showing the increase in cell area by CLP36-knockdown. Cell areas were measured for at least 100 cells per each cell line. Values are means ± standard error. *$p < 0.001$ compared with wild-type cells.

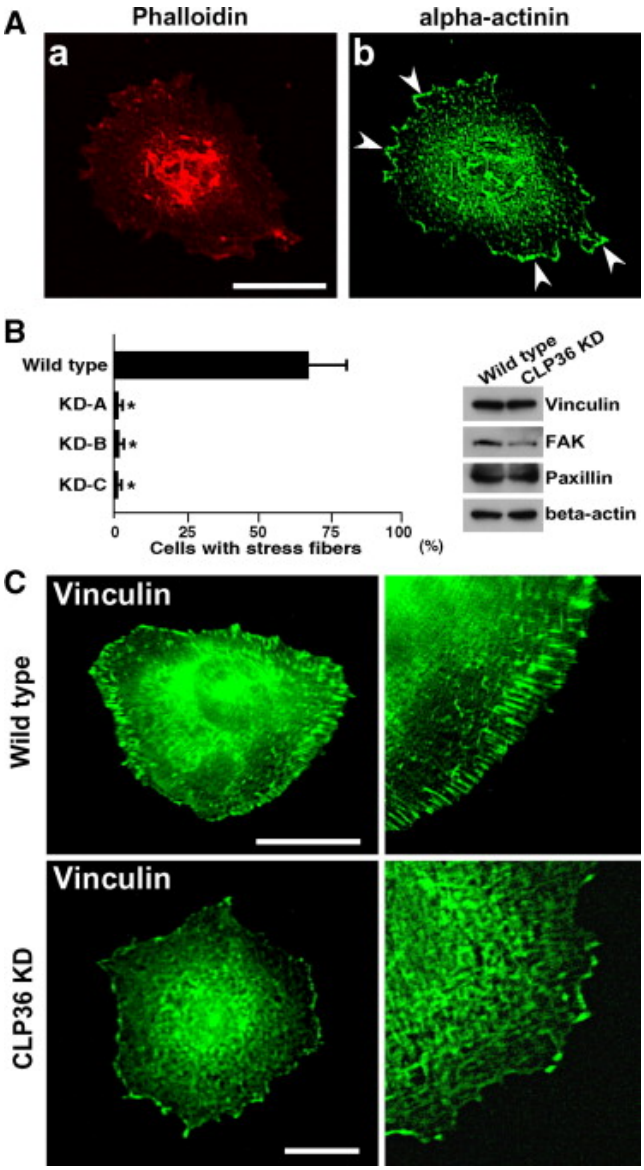
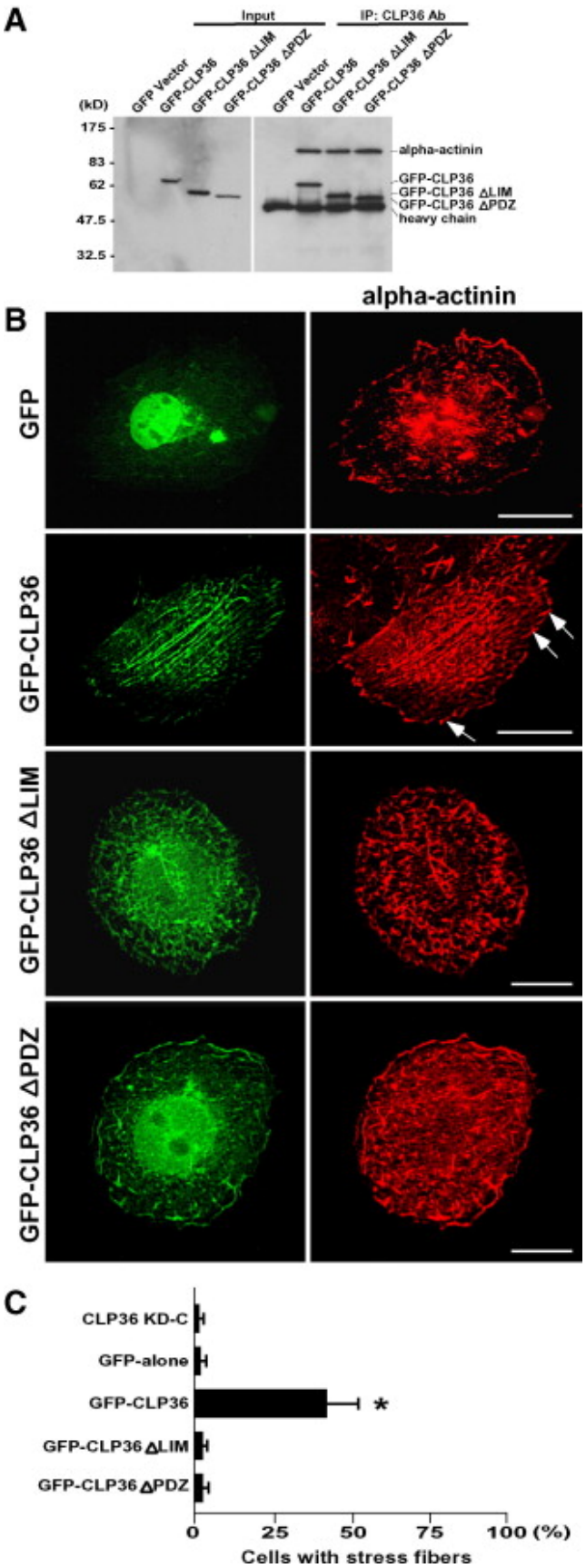


Fig. 3. Depletion of CLP36 impairs the assembly of stress fibers and focal adhesions. (A) CLP36-knockdown cells displayed the loss of stress fiber formation. Alpha-actinin was concentrated at the leading edge of membrane ruffles in the knockdown cells (Ab, arrowheads). Bar, 50 μm. (B) Histograms showing the percentage of cells with stress fibers. Values are means ± standard error for 100 cells per each cell line. *$p < 0.001$ compared with wild-type cells. (C) Vinculin staining indicating the assembly of focal adhesions in wild-type BeWo cells and dot-like focal complexes in CLP36-knockdown cells. Bars, 50 μm. (D) Western-blot analysis showing the expression of focal adhesion proteins (vinculin, FAK, and paxillin) after CLP36-knockdown. Their expressions were not changed after CLP36-knockdown.


A
Input
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GFP Vector
GFP-CLP36
GFP-CLP36 ΔLIM
GFP-CLP36 ΔPDZ
(kD)
175
83
62
47.5
32.5
alpha-actinin
GFP-CLP36
GFP-CLP36 ΔLIM
GFP-CLP36 ΔPDZ
heavy chain
B
alpha-actinin
GFP
GFP-CLP36
GFP-CLP36 ΔLIM
GFP-CLP36 ΔPDZ
C
CLP36 KD-C
GFP-alone
GFP-CLP36
GFP-CLP36 ΔLIM
GFP-CLP36 ΔPDZ
*
0
25
50
75
100 (%)
Cells with stress fibers

Fig. 4. PDZ and LIM domains are both responsible for the assembly of stress fibers and focal adhesions. CLP36-knockdown cells were transfected with GFP-CLP36 or its deletion mutants lacking the PDZ domain (GFP-CLP36 ΔPDZ) or LIM domain (GFP-CLP36 ΔLIM). (A) At 72 h after transfection, cells were lysed and subjected to immunoprecipitation with anti-CLP36 antibody. GFP-CLP36 and its mutants formed a complex with alpha-actinin in the transfected cells. Membranes were probed with anti-CLP36 antibody (left) or anti-CLP36 and anti-alpha-actinin antibodies (right). (B) Transfected cells were stained with anti-alpha-actinin antibody. Addition of GFP-CLP36 recovered stress fiber formation and dash-like focal adhesions at the end of the stress fibers (arrows). In contrast, addition of the mutant forms did not induce stress fiber formation. Bars, 50 μm. (C) Histograms showing the percentage of cells with stress fibers (means ± standard error). Data were collected from four independent experiments, where 100 transfected cells were counted per each construct. $*p < 0.001$, compared with CLP36-knockdown cells (KD-C).