The ZASP-like Motif in Actinin-associated LIM Protein Is Required for Interaction with the α-Actinin Rod and for Targeting to the Muscle Z-line*

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The Z-line is a specialized structure connecting adjacent sarcomeres in muscle cells. α-Actinin cross-links actin filaments in the Z-line. Several PDZ-LIM domain proteins localize to the Z-line and interact with α-actinin. Actinin-associated LIM protein (ALP), C-terminal LIM domain protein (CLP36), and Z band alternatively spliced PDZ-containing protein (ZASP) have a conserved region named the ZASP-like motif (ZM) between PDZ and LIM domains. To study the interactions and function of ALP we used purified recombinant proteins in surface plasmon resonance measurements. We show that ALP and α-actinin 2 have two interaction sites. The ZM motif was required for the interaction of ALP internal region with the α-actinin rod and for targeting of ALP to the Z-line. The PDZ domain of ALP bound to the C terminus of α-actinin. This is the first indication that the ZM motif would have a direct role in a protein-protein interaction. These results suggest that the two interaction sites of ALP would stabilize certain conformations of α-actinin 2 that would strengthen the Z-line integrity.

The muscle Z-line is a highly specialized structure between adjacent sarcomeres in muscle fibers that maintain the organization of the contractile machinery (for review, see Ref. 1). α-Actinin is one of the major components at the Z-line. The α-actinin polypeptide is composed of an N-terminal actin binding domain, four spectrin repeats that form the central rod region (2, 3), and two pairs of C-terminal EF-hands (4). α-Actinin forms an antiparallel homodimer. α-Actinin cross-links actin filaments from opposite sarcomeres to the Z-line and, therefore, has a major mechanical function in keeping the sarcomeres together.

Several PDZ-LIM proteins have been detected in the muscle Z-line and shown to interact with α-actinin (5–12). PDZ domain is a widely expressed protein-protein interaction domain (for review, see Ref. 13). PDZ-LIM proteins form one subgroup of PDZ proteins (13) and are regarded as mediators between cytoskeletal structures and signaling cascades.

PDZ-LIM proteins can be divided in two subclasses; they either contain one or three LIM domains. Actinin-associated LIM protein (ALP)1 (5, 10), C-terminal LIM domain protein (CLP36) (14) (also called hCLIM1 (15) or Elfin (16)), Reversion-induced LIM protein (RIL) (17), and Mystique (Uniprot accession number Q7Z584) belong to the first class, which has one N-terminal PDZ domain and one C-terminal LIM domain. ALP is expressed in muscle (5, 10), whereas CLP36 and RIL are mainly expressed in nonmuscle tissues (11, 18, 19). CLP36 shows also high expression levels in muscle (7, 15, 16). Enigma, Enigma homology protein, and ZASP/Cypher/Oracle form the second class, with one N-terminal PDZ domain and three C-terminal LIM domains. They all are expressed mainly in muscle (6, 9, 20–22). An interesting feature of these seven PDZ-LIM proteins is that ALP, CLP36, and ZASP contain a conserved region, named ZASP-like motif (ZM) (SMART prediction (23) accession number SM 00735, Interpro 006643), in the internal region between the PDZ and LIM domain. This motif is composed of 26 conserved residues.

Several studies have described interactions between the PDZ-LIM proteins and α-actins. ALP, CLP36, Enigma homology protein, and ZASP colocalize at the Z-line with α-actinin (5, 7–9). Also Enigma localizes to the Z-lines and interacts with skeletal β-tropomyosin (22). The interaction between rat ALP and α-actinin 2 has been reported to be mediated via the PDZ domain and the spectrin repeats of α-actinin 2 (10). CLP36 has been described to interact with the spectrin repeats of nonmuscle α-actinin isoforms (α-actinin 1 and α-actinin 4) either via PDZ domain (11) or via the internal region between the PDZ and LIM domains (12). Also the LIM domain (amino acids 129–329) of CLP36 is shown to interact with the C-terminal EF-hands of α-actinin 2 (7). ZASP PDZ domain recognizes the EF region of α-actinin 2 (6, 24), and also, RIL and Enigma homology protein associate with α-actinin via the PDZ domains (9, 25).

ALP-deficient mice develop cardiomyopathy (26), but it is not clear how ALP functions in muscle. To better understand how ALP functions, we have characterized its interaction sites with α-actinin in more detail. Here we show that there are the two interaction sites between human skeletal muscle ALP and α-actinin 2. ALP PDZ domain recognized the C terminus of α-actinin 2, whereas the ZM motif was required for ALP interaction with the rod region of α-actinin. Moreover, we show that the ZM motif was necessary for the recruitment of ALP to the Z-lines in cultured myoblasts. The two interaction sites suggest that ALP may regulate actin filament cross-linking by stabilizing the hinge region between the head and rod regions of α-actinin.

ZASP, Z band alternatively spliced PDZ-containing protein; ZM, ZASP-like motif; CLP36, C-terminal LIM domain protein; SPR, surface plasmon resonance; GFP, green fluorescent protein; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; TRITC, tetramethylrhodamine isothiocyanate; F-actin, filamentous actin; RIL, reversion-induced LIM protein; CHO, Chinese hamster ovary.

Received for publication, February 20, 2004, and in revised form, April 2, 2004
Published, JBC Papers in Press, April 14, 2004, DOI 10.1074/jbc.M401871200

26402 This paper is available on line at http://www.jbc.org

* This work was funded by Academy of Finland Research Grants 51863 and 105211. The costs of publication of this article were defrayed...
**TABLE I**

Plasmid constructs used in this study

| cDNA               | Construct | Amino acids | Vector                          | Cloning site  |
|--------------------|-----------|-------------|---------------------------------|---------------|
| Human ALP, AF039018| PDZ+1     | 1–284       | Modified pET24d                 | BsmBI-NotI    |
|                    | I         | 112–284     | Modified pET24d                 | BsmBI-NotI    |
|                    | PDZ       | 1–84        | Modified pET24d                 | BsmBI-NotI    |
|                    | PDZ+1     | 1–256       | Modified pET24d                 | NotI-NotI     |
|                    | I         | 117–256     | Modified pET24d                 | NotI-NotI     |
|                    | PDZ1      | 1–85        | Modified pET24d                 | NotI-NotI     |
|                    | PDZ2      | 1–119       | Modified pET24d                 | NotI-NotI     |
| Human CLP36, NM_020992| 284 pELGFP (29) | 2–894 | pET8c (28) | XhoI-MluI     |
|                    | EF3–4     | 2–820       | pET8c (2)                       | XhoI-MluI     |
|                    | Rod       | 274–746     | Modified pET24d                 | NotI-NotI     |
|                    | EF3–4     | 821–894     | Modified pET24d                 | NotI-NotI     |
|                    | EF3–4C    | 821–890     | Modified pET24d                 | NotI-NotI     |
| Human ALP, AF039018| PDZ+1     | 1–284       | pELGFP                          | XhoI-NotI     |
|                    | I         | 110–284     | pELGFP                          | XhoI-NotI     |
|                    | PDZ       | 1–112       | pELGFP                          | XhoI-NotI     |
|                    | PDZ+1     | 1–284       | pEGFP-C1                        | EcoRI-BamHI   |
|                    | I         | 110–284     | pEGFP-C1                        | EcoRI-BamHI   |
|                    | PDZ       | 1–112       | pEGFP-C1                        | EcoRI-BamHI   |
|                    | 1+LIM     | 110–364     | pEGFP-C1                        | EcoRI-BamHI   |
|                    | LIM       | 283–364     | pEGFP-C1                        | EcoRI-BamHI   |
|                    | PDZ+2M    | 1–232       | pEGFP-C1                        | EcoRI-BamHI   |
| Chicken ALP, AJ249218| PDZ+1     | 1–241       | pEGFP-C1                        | XhoI-BamHI    |
|                    | I         | 110–241     | pEGFP-C1                        | XhoI-BamHI    |
|                    | PDZ       | 1–110       | pEGFP-C1                        | XhoI-BamHI    |
|                    | PDZ       | 1–110       | Myc-pcDNA3                      | BsmBI-NotI    |

* This construct contains an extra Val before the Stop codon.

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**EXPERIMENTAL PROCEDURES**

**Generation of Human ALP, CLP36, and α-Actinin 2 Constructs—**A cDNA of human skeletal muscle ALP corresponding to the full-length protein (amino acids 1–364) (AF039018) was amplified from the human skeletal muscle cDNA library (Matchmaker, BD Biosciences, Clontech). This was used as a template to generate shorter fragments of ALP. A plasmid containing full-length human CLP36 (11) (NM_020992) obtained from Tea Vallenius (University of Helsinki, Finland) was used as a template to generate shorter fragments of CLP36. ALP and CLP36 obtained from Tea Vallenius (University of Helsinki, Finland) was used as a template to generate shorter fragments of ALP. A PCR fragment containing an N-terminal His6 tag followed by a tobacco etch virus protease recognition site (27) and three extra residues (ENLYFQ) was amplified by PCR and introduced to pcDNA3 vector (Invitrogen) with 0.4 mM isopropyl-1-thio-galactopyranoside. An N-terminal His tag was used for protein purification by nickel nitritolactric acid-agarose (Qiagen). The tag was removed by tobacco etch virus protease (Invitrogen) cleavage either in overnight dialysis against 20 mM Tris-HCl, pH 8.0, at +4°C, or buffer change using PD10 columns (Amersham Biosciences), and proteolysis was performed overnight at 23°C. Anion exchange chromatography (ProteinPak Q 8HR columns, Waters) was used for further purification. Size exclusion chromatography was performed in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, using Superdex 200 16/60 column (Amersham Biosciences). The molecular weight standards for the gel filtration were from Bio-Rad (catalog number 151-1901).

**Surface Plasmon Resonance Detection—**Biacore 3000 system (Biacore, Uppsala, Sweden) was used for surface plasmon resonance (SPR) analysis. Purified protein fragments of ALP and CLP36 were covalently bound from their amino groups to the gold sensor chip surface (CM5) and used as ligand proteins. α-Actinin fragments were used as analyte proteins in the fluid phase, with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.005% surfactant P20 (BR 1000–54, Biacore AB, Uppsala, Sweden) as the running buffer. The Bioevaluation (Biacore) program, assuming 1:1 Langmuir binding, was used for kinetic calculations. SDS-PAGE analysis of proteins used in Biacore is shown in Fig. 1A. Low molecular weight marker was from Amersham Biosciences.

**Fimlamentous Actin (F-Acetin) Sedimentation Assay—**Monomeric actin (Cytokeleton Inc, Denver, CO) was polymerized in the general polymerization buffer (1 mM ATP, 50 mM KCl, 5 mM Tris-HCl, pH 8.0, and 0.5 mM dithiothreitol, according to Cytokeleton Inc.) but in the absence of divalent cations (Ca²⁺ and Mg²⁺) and in the presence of 5 mM EDTA. Polymerization was allowed to continue for 30 min at +23°C. Purified full-length α-actinin 2 (1 µg) and ALP PDZ+1 (3.8 µg) were added to the reaction and mixed carefully. After 30 min of incubation the samples were ultracentrifuged (90,000 rpm, 30 min, +18°C, TLA100 rotor, Beckman Coulter, Buckinghamshire, UK), and supernatants and pellets were separated on 12% SDS-PAGE. The gels were stained with tricfluoroacetic acid and further ether-precipitated and lyophilized. The purity of the peptide was 98%, and the molecular mass was 1041 as expected.
Coomassie Brilliant Blue. Intensities of protein bands were quantified using the QuantityOne program (Bio-Rad). Results of three experiments were used in GraFit 5.0.6 (Erichthum Software Ltd.) to calculate $K_v$ values for $\alpha$-actinin and F-actin interaction.

The molar ratio of ALP PDZ +1 and $\alpha$-actinin 2 in pellet fractions in increasing concentrations of actin (0–10 μM) was calculated using formula, molar ratio of ALP PDZ + $\alpha$-actinin 2 = $S_0 - S_1 - (U_0 - U_1)/$ $(A_0 - A_1)$, where $S$ denotes specific ALP pelleting, $U$ denotes unspecific ALP pelleting, $S_0$ is mol of ALP pelleting in a given actin concentration in constant $\alpha$-actinin 2 concentration, $S_1$ is mol of ALP pelleting in the absence of actin in constant $\alpha$-actinin 2 concentration, $U_0$ is mol of ALP pelleting in given actin concentration without $\alpha$-actinin 2, $U_1$ is mol of ALP pelleting in the absence of actin and without $\alpha$-actinin 2, $A_0$ is mol of $\alpha$-actinin 2 pelleting in given actin concentration in constant ALP concentration, and $A_1$ is mol of $\alpha$-actinin 2 pelleting in the absence of actin in constant ALP concentration. In the calculations $\alpha$-actinin 2 was assumed to be a dimer ($M_r = 207,800$) and ALP PDZ +1 a monomer ($M_r = 30,400$).

**Transfection of CHO and C2C12 Cells—**Chinese hamster ovarian (CHO-K1) cells (ATCC CCL-61, American Tissue Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 units/ml penicillin, and 1 g/ml streptomycin (Sigma-Aldrich). CHO cells were transfected at 90–95% confluence on 6-well plates using 4 μl of LipofectAMINE 2000 (Invitrogen) per well in the absence of serum and antibiotics for 6 h. Serum-containing media was added to cells for overnight incubation. 24 h after transfection cells were plated on coverslips coated with 30 μg/ml fibronectin (Sigma-Aldrich) and blocked with 1% bovine serum albumin in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4). After 4 h cells were fixed in 4% parafomaldehyde, pH 7.4, and 0.5% Triton X-100 on ice for 10 min and washed once with phosphate-buffered saline.

Mouse myogenic cells C2C12 (ATCC, CRL-1772) were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.15% sodium bicarbonate (Sigma-Aldrich). Undifferentiated C2C12 cells were cultured on coverslips and transfected when 50–60% confluent using 4 μg of DNA and 10 μl of LipofectAMINE 2000 per well in a 6-well plate. During transfection no FBS or antibiotics were added to culture media. Media was changed to DMEM with 10% FBS, 1 mM sodium pyruvate, and 0.15% sodium bicarbonate 6 h after transfection. The next day media was changed to DMEM, 10% FBS, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, and antibiotics. After 4–6 h the media was changed to differentiation media: DMEM, 2% horse serum (Sigma-Aldrich), 1 mM sodium pyruvate, antibiotics, and 0.15% sodium bicarbonate. Cells were allowed to differentiate for 5 days. Differentiation media was changed at least twice. Cells were fixed in 4% paraformaldehyde, 0.5% Triton X-100, phosphate-buffered saline, pH 7.4, on ice for 10 min and washed with phosphate-buffered saline.

**Antibodies—**Actin fibers were stained with 0.004 units/ml rhodamine-phallolidin (R-415, Molecular Probes, Invitrogen) 30 min at 23 °C. Fluorescein isothiocyanate-conjugated c-Myc 9E10 antibody (sc-40, Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used to detect the localization of Myc-tagged proteins. Sarcomeric $\alpha$-actinin was stained using sarcomeric $\alpha$-actinin-specific antibody (A7811, Sigma-Aldrich) as a primary antibody and TRITC-conjugated anti-mouse IgG as a secondary (115–025-075, Jackson Immunoresearch Laboratories, West Grove, PA). Vinculin was stained using VIIF9 primary antibody (30) and TRITC-conjugated anti-mouse IgG as a secondary antibody.

**RESULTS**

**ALP and $\alpha$-Actinin 2 Have Two Interaction Sites—**To clarify the conflicting literature about the interactions between PDZ-LIM proteins and $\alpha$-actinin (see the Introduction for references), we studied the interaction of purified recombinant protein fragments by surface plasmon resonance (SPR). We found that the PDZ domains of ALP (amino acids 1–84) and CLP36 (amino acids 1–85) interacted with the C-terminal EF-hands (EF3-4) of human $\alpha$-actinin 2 (Table II). The C terminus of $\alpha$-actinin 2 contains the sequence ESDL, which fits with the consensus ligand sequence of group 1 PDZ domains (S/T/XΦ-COOH (X is any amino acid, Φ is a hydrophobic amino acid) (for review, see Ref. 13)). The deletion of this sequence from the EF3-4 fragment abolished the interaction, and a synthetic peptide containing the sequence of the last 10 residues of $\alpha$-actinin 2 (SSALYGESDL) inhibited the interaction in a concentration-
dependent manner (Fig. 2A). The apparent $K_d$ values for the interaction of ALP PDZ-(1–84) and CLP36 PDZ-(1–85) with EF3-4 were 8.6 μM (S.E. ± 1.02 μM) and 15.0 μM (S.E. ± 0.62 μM), respectively.

Although we could measure the interaction of ALP and CLP36 PDZ domains with the EF3-4 fragments, we could not directly show their interaction with the full-length α-actinin 2. To test whether this was caused by steric hindrance on the SPR surface, we tested the interaction with longer PDZ domain constructs. CLP36 PDZ-(1–119) interacted with the full-length α-actinin 2 (Table II) but not with the full-length α-actinin lacking the last two EF-hands (ΔEF3-4, Table II). Size exclusion chromatography confirmed that the recombinant full-length α-actinin 2 (not shown) and ΔEF3-4 proteins were dimers (Fig. 1B). In competitive assays, the short ALP PDZ-(1–84) and CLP36 PDZ-(1–85) inhibited the interaction of CLP36 PDZ-(1–119) with the full-length α-actinin 2 (Fig. 2B), although the inhibition by ALP PDZ-(1–84) was weaker when compared with CLP36 PDZ-(1–85). Thus, our results showed that the PDZ domains of ALP and CLP36 interact with the C-terminal peptide of α-actinin 2.

In addition to the interaction mediated by the PDZ domains, the internal regions (named I) of ALP and CLP36 interacted with the rod region of α-actinin 2 (Fig. 2C and Table II). In this study we used the rod fragment composed of four spectrin repeats that form a stable dimer (2) and should not contain exposed hydrophobic surfaces. SPR experiments revealed apparent $K_d$ values of 6.8 μM (S.E. ± 0.82 μM) and 1.3 μM (S.E. ± 0.03 μM) for the internal regions of ALP I (residues 112–284) and CLP36 I (residues 117–256), respectively. We hypothesized that ZM motif might be involved in the internal recognition of the rod region because this motif is present both in ALP and CLP36. Indeed, ALP PDZ+ZM-(1–232) interacted with the rod region of α-actinin 2 (Fig. 2D; Table II). Taken together, our SPR results suggested that there are two interaction sites between these PDZ-LIM proteins and α-actinin 2. The PDZ domains interact with the C terminus of α-actinin, and the ZM motif in the internal region of ALP is involved in the interaction with the rod region of α-actinin.

**ALP Does Not Affect the Ability of α-Actinin 2 to Bind F-actin**—After finding the two interaction sites we wanted to see if the binding of ALP would affect the ability of α-actinin 2 to bind F-actin. To avoid the effects of Zn$^{2+}$ binding LIM domain in actin polymerization, ALP PDZ+I (residues 1–284), containing the PDZ domain and the internal region, was used in this assay. Still, this protein had a tendency to nonspecifically sediment with F-actin, but this could be avoided by performing the assay in the absence of Mg$^{2+}$ and Ca$^{2+}$ and in the presence of 5 mM EDTA. Actin polymerization is not affected markedly in the absence of divalent cations (31). Under these conditions, the amount of ALP in pellet alone with actin increased from 2% (0 μM actin) to 13.4% (10 μM actin). When α-actinin 2 was included in reactions, the amount of ALP in the pellet increased from 9.7 to 33.6%. The calculation of mole quantities from densitometric scans of ALP PDZ+I to α-actinin 2 in the pellet gave the average stoichiometry of 1.6 (range 1.0–2.2). This value is consistent with 2:1 stoichiometry of ALP/α-actinin 2 interaction. However, the presence of ALP had no significant effect on the interaction of α-actinin 2 with F-actin (Fig. 3). The apparent $K_d$ with ALP was 1.1 μM (S.E. ± 0.18 μM) and without ALP was 0.93 μM (S.E. ± 0.10 μM). These values are similar as shown previously (32). Thus, these experiments suggest that a dimer of α-actinin 2 interacts with two ALP monomers and that this binding has no effect on α-actinin 2-actinin interaction.

**ALP Internal Region Alone or Together with PDZ Domain Localizes to Stress Fibers**—To study if the two interactions observed with purified proteins can take place in living cells, GFP constructs of human ALP were transfected to CHO cells. Human ALP PDZ+I-(1–284) (Fig. 4, a–c), the internal region I (110–284) alone (Fig. 4, d–f), and I+LIM-(110–364) (Fig. 4, g–i) localized to stress fibers in cultured CHO cells, but ALP PDZ-(1–112) (Fig. 4, j–l) or LIM domain alone (not shown) did not. Both PDZ and LIM showed strong a nuclear signal, similar to pEGFP vector alone. In undifferentiated C2C12 cells, GFP-ALP construct showed similar localization along stress as in CHO cells (results not shown). We did not observe GFP-ALP colocalization with vinculin to focal adhesions (Fig. 4, m–o); rather, it appeared the GFP-ALP was excluded from the vinculin-positive structures. The chicken ALP is reported to colocalize with vinculin (5), whereas the human CLP36 has been shown not to (12). This apparent discrepancy may be caused by subtle experimental differences.

Because PDZ domain alone did not localize, we wanted to study if the PDZ+I and I would have differences in the localization in CHO cells (Fig. 5). This experiment suggested that PDZ+I would localize more efficiently to stress fibers than I region alone, but no significant difference was observed between PDZ+I and I+LIM. Thus, it is not clear whether the difference between I and PDZ+I would reflect the contribution of PDZ domain in the interaction with α-actinin or whether the longer constructs would just be more stable than the I region alone.

We tested both N- and C-terminal added GFP together with the ALP PDZ domain, but neither of them localized to actin stress fibers in CHO cells. To find out whether the GFP tag prevents the localization of the PDZ domain, we generated PDZ domain constructs with an N-terminal Myc epitope. The Myc-PDZ domain of human ALP and chicken (smooth muscle) ALP were tested in CHO cells, but neither of them localized to stress fibers (human ALP PDZ Fig. 6, d–f). A Myc-tagged full-length ALP was used as a positive control, and it localized clearly to stress fibers (Fig. 6, a–c). PDZ domains showed mainly strong nuclear localization, and also, some cells transfected with full ALP had a strong nuclear signal in addition of stress fiber localization.

**Table II**

Summary of SPR interaction results

| Protein          | α-Actinin 2 | ΔEF3-4 | RI-R4 | EF3-4 | EF3-4S | C-peptide |
|------------------|------------|--------|-------|-------|--------|-----------|
| ALP PDZ+I, 1–284| +          | +      | +     | +     | +      | NT*       |
| CLP36 PDZ+I, 1–256| +        | +      | +     | +     | +      | NT        |
| ALP I, 112–284  | +          | +      | +     | +     | +      | NT        |
| CLP I, 117–256  | +          | +      | +     | +     | +      | NT        |
| ALP PDZ, 1–84   | +          | +      | +     | +     | +      | NT        |
| CLP PDZ, 1–85   | +          | +      | +     | +     | +      | NT        |
| ALP PDZ, 1–119  | +          | +      | +     | +     | +      | NT        |
| ALP PDZ+ZM, 1–232| NT       | NT     | +     | +     | +      | NT        |

* NT, not tested.
* Measured by competitive assay.
localization. Thus, although the ALP PDZ domain interacted with α-actinin, we could not show their colocalization in CHO cells.

We showed above that the internal region of ALP was sufficient for targeting to stress fibers. To characterize more precisely the requirement of sequences around the ZM motif in the
Localization, we generated Myc-tagged fragments of the internal region (named H9004 I1–5) and transfected them into CHO cells. H9004 I1-(112–232) containing the ZM motif localized along stress fibers (Fig. 6, g–i), but PDZ (j–l) did not. ALP did not colocalize with vinculin at focal adhesions (m–o). h, human.

Fig. 4. Localization of GFP-ALP in CHO cells. GFP fluorescence is shown on the left. F-actin was stained with rhodamine-phalloidin (middle). Merged images are shown on the right. ALP PDZ+I, I and I+LIM localized along stress fibers (a–i), but PDZ (j–l) did not. ALP did not colocalize with vinculin at focal adhesions (m–o).

Fig. 5. Quantification of the recruitment of ALP GFP constructs to stress fibers in CHO cells. % of cells having stress fiber-like GFP localization and p values from Student’s t test are indicated. ALP PDZ+I and ALP I had a C-terminal GFP tag, and I+LIM had an N-terminal GFP. Two persons counted the number of cells showing actin filament-associated fluorescence (in 100 transfected cells/sample) from 10 coded samples from each transfection.

Fig. 6. Localization of Myc-tagged ALP constructs in CHO cells. The anti-Myc staining is shown in the left panel, and rhodamine-phalloidin is shown in the middle panel. Merged images are shown on the right. Full ALP localized along stress fibers (a–c), but PDZ did not (d–f). Internal constructs ΔI1-(112–232) and ΔI5-(151–263) localized to stress fibers (g–i and m–o), but ΔI2-(112–181) did not (j–l). A summary of the localization of all Myc-tagged constructs tested is shown in panel p.

region was excluded but still contained the ZM motif, did not (summarized in Fig. 6p). Taken together, these results suggested that the ZM motif is necessary for localization of ALP to stress fibers, and the amino acids 151–232 would be the minimal sequence.

Skeletal and Smooth Muscle ALP Localize to Z-lines in a Similar Manner—Because ALP is localized at the Z-lines in differentiated muscle cells and the ALP and ZASP/Cypher gene depletion in mice may abrogate Z-line stability or turnover (24, 26), we wanted to study the requirement of the two interaction sites also in the Z-line localization. GFP constructs of ALP were transfected to mouse C2C12 myoblasts. The differentiation of these cells toward myotubes can be induced by media containing 2% horse serum. Localization to Z-lines was detected after 5 days of differentiation by double staining with a-actinin. Human ALP PDZ+I-(1–284) (Fig 7, a–c), ALP I+LIM-(110–364) (Fig 7, d–f), and the I region (110–284) (not shown) colocalized with a-actinin at the Z-
yeast two-hybrid screen (10), it does not completely prevent ALP localization to the Z-lines in cardiomyocytes (33). Even though all isoforms of α-actinin contain the consensus sequence for PDZ binding, before this report only the PDZ domain of ZASP/Cypher and one of the PDZ domains of an unrelated protein MAGI-1 were shown to interact with the C-terminal α-actinins (6, 34). The internal region of ZASP has been reported not to interact with α-actinin 2, but it still localizes to the Z-line (8).

Although our biochemical studies clearly showed that the PDZ domains of ALP and CLP36 interacted with the C-terminal peptide of α-actinin, the ALP PDZ domain alone was not able to colocalize with α-actinin in cell culture models. There are several possible reasons for this discrepancy. First, although this and other studies (6, 8–11) suggest that α-actinin interaction is a common property of many PDZ domains of the PDZ-LIM protein family, the affinities of interaction may vary. Second, in vivo, a sequential interaction mechanism may be required so that the ZM motif first binds to α-actinin, and after that, the PDZ domain can interact. Third, the C terminus of α-actinin may be engaged in other interactions that may be either intermolecular or intramolecular, and these interactions may be regulated locally. Indeed, the interaction site of α-actinin with titin is localized close to the C terminus (35); intramolecular interactions between the two most C-terminal EF-hands and the rod region sequences close to the hinge region have been shown to compete with the titin interaction, and these interactions are regulated by phosphatidylinositol 4,5-bisphosphate and other phospholipids (28).

What might be the function of the two interaction sites between α-actinin 2 and ALP or CLP36? We showed that ALP binding does not change the affinity of α-actinin F-actin interaction. Interestingly, the interaction sites of ALP on α-actinin reside on different sides of the hinge region, which is the main flexible part in the molecule. Thus, if the two interaction sites are occupied by a single molecule of ALP or CLP36, the hinge region of α-actinin may be stabilized. This would restrict the possible F-actin cross-linking geometries of α-actinin. It is known that α-actinin can cross-link F-actin in a wide variety of geometries that can even differ as much as 180° (36). However, only a single geometry of cross-linking is observed in the muscle Z-line, where α-actinin cross-links only antiparallel actin filaments coming from opposite sarcomeres, not parallel ones coming from a single sarcomere (37). This organization of α-actinin in the Z-line is consistent with its major function in providing the mechanical link between sarcomeres. If α-actinin would also cross-link parallel actin filaments in the Z-line, the mechanical strength of the structure would be compromised. We propose that one of the functions of the two binding sites of ALP and α-actinin might be to keep α-actinin in the antiparallel cross-linking conformation and, thus, provide mechanical strength to the Z-lines. ALP knockout mice showed dilated cardiomyopathy (26), and according to our hypothesis, this could be caused by the presence of cross-links between parallel actin filaments instead of antiparallel. We would also predict that ZASP/Cypher would have similar interaction sites with α-actinin and, thus, similar function. The ZASP/Cypher knockout mice have even more severe cardiomyopathy and muscle dystrophy phenotypes than the ALP mice (24). The differences in ALP and ZASP/Cypher phenotypes may mirror their specific functions in different muscle types.

The ZM Motif Is Required for Z-line Targeting—We showed that constructs containing the ZM motif of ALP localized to the Z-line in cultured myoblasts. On the other hand, a construct lacking this motif failed to localize to the Z-lines. More detailed
analysis was done in CHO cells, and the ZM motif was found to be necessary and sufficient for localization of ALP fragments to stress fibers. In addition, our biochemical data suggest that the ZM motif of ALP is required for the interaction with the rod region of α-actinin 2.

The ZM motif is a 26-residue conserved sequence found in some of the PDZ-LIM proteins: ALP, CLP36, and ZASP/Cypher (Fig. 8A). No other matches of this motif can be found in vertebrates by using the SMART server (23) (smart.embl-heidelberg.de, version 4.0, database Jan 28, 2004). Interestingly there are two alternatively spliced exons in human ALP and ZASP/Cypher (Fig. 8) (exons 4 and 6). In chicken the transcript containing the sequence resembling exon 4 is called the skeletal muscle isoform of ALP and exon 6 resembles the smooth/cardiac muscle ALP. On the contrary, in mouse ZASP/Cypher exon 4 is reported to be cardiac-specific and exon 6 skeletal muscle-specific (38). In human, the alternative splicing appears to be less tissue-specific, and both exon 4- and exon 6-containing transcripts are found in heart (39). In this study we used the human exon 4-containing ALP variant and chicken smooth muscle ALP resembling human exon 6 and showed that they both can localize to the Z-line. Currently we have no information about the different biochemical functions of the two ALP or ZASP/Cypher ZM-encoding exons. The ZM motif might be a signature in an independently folded domain. The heterologously expressed internal region of ALP is quite stable, and it can be purified in microgram amounts. Thus, it appears that it can fold independently of the PDZ and LIM domains. Our cell culture data suggest that residues 151–232 would be the minimal peptide required for the folding of the ZM motif.

Our results suggest that the ZM motif is important for the function of human ALP and that it would be needed for the interaction with α-actinin 2 in the muscle Z-line. We propose that the ZM motif might have a similar function also in at least ZASP, which is also found in the Z-line. This hypothesis is supported by the recent finding of mutations in both of the ZM-encoding exons of ZASP in dilated cardiomyopathy patients (39). The mutations in exon 4 are Ser to Leu and Thr to Ile substitutions in the ZM consensus (Fig. 8C). Two mutations have been reported in exon 6; one is a Lys to Met substitution 20 residues before the consensus sequences, and the other one is located farther upstream. Vatta et al. (39) suggest that the mutations in exon 6 might have a direct effect in the interaction of ZASP with actin network. Our results give the first direct evidence that the ZM motif is indeed involved in the interaction of the PDZ-LIM family proteins with α-actinin. This interaction may have a role in the development of dilated cardiomyopathy.

Acknowledgments—We thank Päivi Pirilä for valuable advice during SPR experiments and Tea Vallenius and Tiila Kiema for critical reading of the manuscript. Tea Vallenius, Michael Way, and Mary Beckerle are acknowledged for the plasmids.

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