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# Purification and Characterization of an α-Actinin-binding PDZ-LIM Protein That Is Up-regulated during Muscle Differentiation*

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**α-Actinin is required for the organization and function of the contractile machinery of muscle. In order to understand more precisely the molecular mechanisms by which α-actinin might contribute to the formation and maintenance of the contractile apparatus within muscle cells, we performed a screen to identify novel α-actinin binding partners present in chicken smooth muscle cells. In this paper, we report the identification, purification, and characterization of a 36-kDa smooth muscle protein (p36) that interacts with α-actinin. Using a variety of *in vitro* binding assays, we demonstrate that the association between α-actinin and p36 is direct, specific, and saturable and exhibits a moderate affinity. Furthermore, native co-immunoprecipitation reveals that the two proteins are complexed *in vivo*. p36 is expressed in cardiac muscle and tissues enriched in smooth muscle. Interestingly, in skeletal muscle, a closely related protein of 40 kDa (p40) is detected. The expression of p36 and p40 is dramatically up-regulated during smooth and skeletal muscle differentiation, respectively, and p40 colocalizes with α-actinin at the Z-lines of differentiated myotubes. We have established the relationship between p36 and p40 by molecular cloning of cDNAs that encode both proteins and have determined that they are the products of a single gene. Both proteins display an identical N-terminal PDZ domain and an identical C-terminal LIM domain; an internal 63-amino acid sequence present in p36 is replaced by a unique 111-amino acid sequence in p40. Analysis of the sequences of p36 and p40 suggest that they are the avian forms of the actinin-associated LIM proteins (ALPs) recently described in rat (Xia, H., Winokur, S. T., Kuo, W.-L., Altherr, M. R., and Bredt, D. S. (1997) *J. Cell Biol.* 139, 507–515). The expression of the human *ALP* gene has been postulated to be affected by mutations that cause facioscapulohumeral muscular dystrophy; thus, the characterization of ALP function may ultimately provide insight into the mechanism of this disease.**

In vertebrates, there are three types of muscle: skeletal, cardiac, and smooth, that contract by an actin- and myosin-dependent mechanism. Locomotion depends on the ability of skeletal muscle to contract rapidly, blood circulation depends on cardiac muscle contraction, and involuntary movements such as peristalsis of the gastrointestinal tract depend on smooth muscle function. In skeletal and cardiac muscle cells, the contractile apparatus is organized into functional units called sarcomeres, each of which is bordered by a structure known as the Z-disc. The Z-discs serve to anchor the actin filaments at the ends of the sarcomere. In smooth muscle cells, the actin- and myosin-rich contractile machinery is organized quite differently; rather than appearing in semicrystalline sarcomeric arrays, the contractile elements are obliquely organized. The smooth muscle contractile apparatus exhibits no Z-discs but instead has two other structures, dense bodies and dense plaques, that are thought to anchor and integrate the actin filaments within the muscle cells (2). Z-discs, dense bodies, and dense plaques thus appear to play parallel, central roles in muscle cytoarchitecture and function. Perhaps not surprisingly, given their similar roles in different muscle types, Z-discs, dense bodies and dense plaques are all enriched in α-actinin, a major structural protein present in all muscle cells (3, 4).

α-Actinin is an actin filament cross-linking protein that exists as an antiparallel homodimer in muscle and nonmuscle cells (5, 6). In nonmuscle cells, α-actinin is found periodically along the actin stress fibers, where it is thought to be involved in bundling actin thin filaments into stress fibers (7). Nonmuscle α-actinin is also present at the ends of the stress fibers, in focal adhesions, where it binds the cytoplasmic domain of the integrin $\beta_1$ subunit, an observation that suggests a molecular mechanism by which α-actinin might link microfilaments to the cell membrane (8, 9). α-Actinin also appears to play a key role in organizing the actin machinery in muscle; for example, high resolution electron microscopic analyses have illustrated that, in the Z-discs of striated muscle, α-actinin forms cross-links that anchor actin filaments (10, 11).

Genetic studies have clarified substantially the central role of α-actinin in muscle structure and function. In *Drosophila*, α-actinin loss-of-function mutations perturb Z-disc integrity and disrupt myofibrillar attachments to tendon cells (12, 13). These structural abnormalities are associated with reduced muscle function and lead to progressive paralysis and larval lethality (12). Certain weaker α-actinin alleles affect the morphology and function of thoracic muscles, leading to a flightless phenotype (12). It appears that, in *Drosophila*, α-actinin is not absolutely required for the assembly of the contractile machinery during development, since embryogenesis proceeds normally. Rather, α-actinin appears to play a critical role in anchoring and stabilizing the contractile filaments against the forces of muscle contraction.

α-Actinin-rich structures also perform critical functions in muscle of the nematode *Caenorhabditis elegans*. In *C. elegans*,

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actin filaments of the body wall muscle cells are attached to the plasma membrane through α-actinin-rich structures called dense bodies (14). The function of the dense bodies resembles that of the Z-lines and the dense plaques of the vertebrate striated and smooth muscles, respectively. Although mutations in the *C. elegans* gene encoding α-actinin have not been described, mutations that affect other dense body constituents have been characterized. For example, nematode dense bodies contain vinculin and worms that lack vinculin function display disorganized muscle and are paralyzed (15). Thus, a defect in the organization of the α-actinin-rich dense bodies compromises muscle cytoarchitecture and function.

Despite the well established and apparently universal importance of α-actinin-rich structures for the subcellular organization and function of diverse muscle types, little is known about other proteins that cooperate with α-actinin in the establishment and maintenance of the contractile machinery. In order to better understand the molecular mechanism by which α-actinin participates in the stabilization of the contractile elements during muscle contraction, we sought to identify novel α-actinin-binding partners. Here we report the identification, purification, and characterization of a 36-kDa α-actinin-binding partner (p36) that is expressed in cardiac and smooth muscle. By a variety of binding studies, we demonstrate that the association of p36 with α-actinin is direct, specific, and saturable. We have also identified a higher molecular weight isoform, called p40, that is expressed exclusively in skeletal muscle and is colocalized with α-actinin at the Z-lines. Furthermore, the expression of both p36 and p40 is induced upon muscle differentiation, raising the possibility that these cytoskeletal PDZ-LIM proteins play a critical role in the organization of actin filament arrays within muscle cells. Characterization of the domain structures of p36 and p40 has revealed the presence of an N-terminal PDZ domain (16) and a C-terminal LIM domain (17) in each protein. Sequence analysis revealed that the proteins described here are likely to be the avian homologues of the actinin-associated LIM protein (ALP),[1] a candidate for the protein affected in facioscapulohumeral muscular dystrophy (1).

## EXPERIMENTAL PROCEDURES

*Protein Purification and Microsequencing*—Frozen chicken gizzards were used to extract avian smooth muscle proteins as described previously (18). Proteins present in the extract were precipitated with increasing amounts of ammonium sulfate: 0–15%, 15–27%, 27–34%, 34–43%, and 43–61% saturation. α-Actinin was purified from the 27–34% ammonium sulfate precipitate (19).

The 36-kDa α-actinin-binding partner, called p36, was purified from the 15–27% ammonium sulfate precipitate. All the different purification steps were performed at 4 °C. The ammonium sulfate precipitate was resuspended in 20 ml of buffer B10 (20 mM Tris acetate, pH 7.6, 10 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol) and dialyzed overnight against buffer B10. The mixture of proteins was loaded on an 11 × 2-cm DEAE-cellulose column (Whatman) equilibrated with buffer B10. The proteins that fail to bind the matrix were applied to a 9 × 2-cm CM-cellulose column (Whatman) equilibrated in buffer B10. The bound proteins were eluted with a 200-ml linear gradient of 0–300 mM KCl prepared in buffer B10.

Purified p36 was electrophoresed on 15% polyacrylamide gels and transferred to polyvinylidine difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). Ponceau S-stained, excised p36 bands were prepared and subjected to proteolytic cleavage using trypsin according to the procedure described by Fernandez *et al.* (20). The proteolytic peptides were resolved by high pressure liquid chromatography using a reverse phase C-18 column (Waters Chromatography Division, Milford, MA). Sequence analysis of intact p36 or individual peptides was performed on a 477A protein sequencer (Applied Biosystems, Inc., Foster City, CA).

*Protein Radioiodination and Blot Overlay Assay*—Purified α-actinin was radiolabeled using Na$^{125}$I (ICN Pharmaceuticals Inc., Costa Mesa, CA) as described previously (19, 21). The purity of the radioiodinated protein was evaluated by SDS-PAGE followed by autoradiography.

Blot overlay assays were performed using the method described previously (19). Briefly, proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and the nitrocellulose strips were incubated for 4 h in the presence of 250,000 cpm/ml of [$^{125}$I]α-actinin. In the competition experiment using radioiodinated α-actinin, a 2,000-fold molar excess of competing protein (α-actinin or BSA) was added to the blot overlay buffer. Nitrocellulose membranes were then subjected to autoradiography at −80 °C with an intensification screen.

*Gel Electrophoresis and Western Immunoblotting*—Protein fractions were separated by SDS-PAGE according to the method of Laemmli (22) except with a bisacrylamide concentration of 0.13%. 12.5 or 15% polyacrylamide gels were used in this paper.

For Western immunoblotting, proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Rabbit polyclonal antibodies raised against chicken p36 (K55) or chicken α-actinin (provided by K. Burridge) were used, followed by horseradish peroxidase linked to protein A (Amersham Pharmacia Biotech). Immunodetection was enhanced using chemiluminescent techniques (ECL, Amersham Pharmacia Biotech).

*Solid-phase Binding Assay*—Solid-phase binding experiments were performed in removable microtiter wells as described previously (21), except that the wells were coated with purified chicken p36 at 0.1 mg/ml. The [$^{125}$I]α-actinin used in these experiments was radioiodinated to a specific activity of $23.5 \times 10^6$ cpm/μg. A constant amount of [$^{125}$I]α-actinin (0.09 pmol) was incubated for 2.5 h in p36-coated wells with increasing amounts of competing proteins, unlabeled α-actinin, or BSA. After washes, the bound counts were determined using a Packard Multi-Prias 1 γ-counter (Packard Instrument Co. Inc., Meriden, CT).

*Determination of Stokes' Radius and Relative Sedimentation Coefficient*—The Stokes' radius of the purified chicken p36 was estimated by calibrated gel filtration chromatography. The purified protein or the gel filtration standards were applied to a Sepharose CL-6B (Amersham Pharmacia Biotech) column (1 m × 1.2 cm), equilibrated in buffer B$^+$ (20 mM Tris acetate, pH 7.6, 140 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol). The gel filtration standards used to calibrate the column were albumin (3.55 nm), ovalbumin (3.05 nm), and myoglobin (1.91 nm) from Amersham Pharmacia Biotech.

The relative sedimentation coefficient of the purified chicken p36 was determined by sucrose density gradient centrifugation as described previously (18). The sucrose gradients were prepared in buffer B$^+$. The standard proteins used in these experiments were albumin (4.3 S), ovalbumin (3.6 S), and carbonic anhydrase (2.8 S) from Bio-Rad.

The native molecular mass and the frictional ratio of p36 were calculated as described by Siegel and Monty (23) using a value of 0.711 cm$^3$/g for the partial specific volume; the partial specific volume of p36 was calculated based on the protein's amino acid sequence described below.

*Cell Culture*—Chicken embryo fibroblasts (CEF) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The C2C12 myogenic cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% horse serum (growth medium). C2C12 differentiation was induced by transferring the cells into Dulbecco's modified Eagle's medium supplemented with 2% horse serum (differentiation medium).

*Antibody Production and Confocal Immunofluorescence Microscopy*—The rabbit polyclonal antibody, K55, was raised against purified chicken p36. Double label indirect immunofluorescence of CEF cells was performed as described previously (24). CEF cells were cultured either on glass coverslips for 24 h (spread cells) or on fibronectin-coated glass coverslips for 15 min (spreading cells). For immunostaining, we used the anti-p36 polyclonal antibody (K55) followed by an FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and an anti-α-actinin monoclonal antibody (ICN Pharmaceuticals Inc.) followed by a Texas Red-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories). C2C12 cells were cultured on glass coverslips for 6 days in differentiation medium, and immunocytochemistry was performed as described by Arber *et al.* (25). The same primary and secondary antibodies used for immunostaining of the CEF cells were used for the C2C12 cells, except that α-actinin was detected with a monoclonal anti-sarcomeric α-actinin antibody (Sigma). Cells were observed on a confocal laser scanning microscope (Bio-Rad) with an optical section height of 1 μm.

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[1] The abbreviations used are: ALP, actinin-associated LIM protein; aa, amino acids; CEF, chicken embryo fibroblasts; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; bp, base pair(s).

*Immunoprecipitation*—Immunoprecipitation experiments were performed as described previously (21). Briefly, CEF cells were lysed in radioimmune precipitation buffer (10 mM Tris, pH 8, 140 mM NaCl, 1% Triton X-100, 0.2% deoxycholate, 0.02% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml phenantholine) and scraped off the dish. After a 30-min incubation on ice, the lysate was centrifuged at 10,000 rpm for 10 min. The supernatant was incubated with protein A-agarose beads (Sigma) for 1 h at 4 °C and centrifuged for 2 min at 2,000 rpm. The supernatant was then incubated for 1 h at 4 °C with either 3 μl of the anti-p36 antibody (K55) or 3 μl of the corresponding preimmune serum, followed by a 1.5-h incubation with protein A-agarose beads. The beads were washed five times with the lysis buffer, resuspended in 30 μl of 2× Laemmli sample buffer, and boiled for 5 min. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western immunoblotting.

*Embryonic Chicken Tissue Lysate Preparation*—Protein extracts were obtained from chicken embryo tissues as described previously (24, 26). Protein samples from 19-day-old chicken embryos were used in the tissue distribution experiment, whereas arteries from 11-, 13-, 15-, and 18-day old chicken embryos were used in the developmental time course experiment. Briefly, 5 ml of distilled $H_2O$ plus 1 mM phenylmethylsulfonyl fluoride was used to homogenized 1 g of each tissue (wet weight). Samples were resuspended in 2× Laemmli sample buffer, and the DNA was sheared. Samples were then boiled for 4 min, and 10 μl of each were loaded onto a gel.

*Isolation of Chicken p36 and p40 cDNAs*—mRNA was isolated from CEF cells using the Oligotex Direct mRNA kit (Quiagen Inc., Santa Clarita, CA). First strand cDNAs were generated from the CEF mRNAs using the T-Primed First-Strand kit (Amersham Pharmacia Biotech). Two degenerate primers were synthesized based on two peptide sequences, GIDFNQ (aa 20–25) and FKPIGTA (aa 112–118), obtained by microsequencing of the purified chicken p36. These degenerate primers were used to amplify a 296-bp DNA fragment from the cDNAs. Bases on the sequence of this fragment, two new primers (CCAGCCTTTGATCATAACCAGG and GCTTGAATTCCTGTGGTTCAGC) that corresponded to internal sequence were synthesized and were used to amplify a 269-bp DNA fragment from the 296-bp fragment. Using the 269-bp DNA fragment, we screened 1,000,000 recombinant phage from a total chicken embryo cDNA library (CLONTECH Laboratories Inc., Palo Alto, CA) and obtained five positive clones. Two of the five positive plaques were isolated, purified, and sequenced on both strands. Sequence analysis reveals that the two cDNAs encode proteins that are identical, except in a central region of the proteins; we conclude that the two cDNAs encode the p36 and p40 isoforms that we have detected immunologically.

## RESULTS

*A Direct and Specific Interaction between α-Actinin and a 36-kDa Protein*—In order to understand more precisely the cellular mechanisms involved in the assembly of the actin-based cytoskeleton during myogenesis, we have initiated an effort to identify α-actinin-binding partners in muscle cells using a blot overlay assay. Proteins extracted from avian smooth muscle were fractionated by precipitation with increasing amounts of ammonium sulfate (0–15, 15–27, 27–34, 34–43, and 43–61% saturation). Proteins present in each of these fractions were resolved by SDS-PAGE (Fig. 1*A*). A similar gel was transferred to nitrocellulose and probed with radioiodinated α-actinin (Fig. 1*B*). The radioiodinated α-actinin interacts directly with two proteins. The 23-kDa protein present primarily in the 34–43% ammonium sulfate precipitate (Fig. 1*B*, *lane 4′*) is CRP1, a LIM protein that we have previously characterized as a binding partner for α-actinin (21, 27). A second polypeptide identified in the screen is present in the 15–27% precipitate (Fig. 1*B*, *lane 2′*) and migrates at a molecular mass of 36 kDa (hereafter called p36). The purity of the α-actinin probe used in this experiment is shown in Fig. 1*C*.

In an effort to analyze the specificity of the α-actinin-p36 interaction, we performed a competition experiment. The smooth muscle-derived proteins contained in the 15–27% precipitate were resolved by SDS-PAGE (Fig. 1*D*). Similar gels were transferred to nitrocellulose, and the nitrocellulose strips were incubated with radioiodinated α-actinin in the absence of competing protein (Fig. 1*E*) or in the presence of an excess of unlabeled α-actinin (Fig. 1*F*) or BSA (Fig. 1*G*). As shown in Fig. 1*B*, the radioiodinated α-actinin interacts directly with p36. Furthermore, the binding of [$^{125}$I]α-actinin to p36 is effectively eliminated in the presence of unlabeled α-actinin but not in presence of an equimolar amount of BSA. Taken together, these experiments show a direct and specific interaction between the actin-binding protein, α-actinin, and a 36-kDa protein expressed in smooth muscle cells.

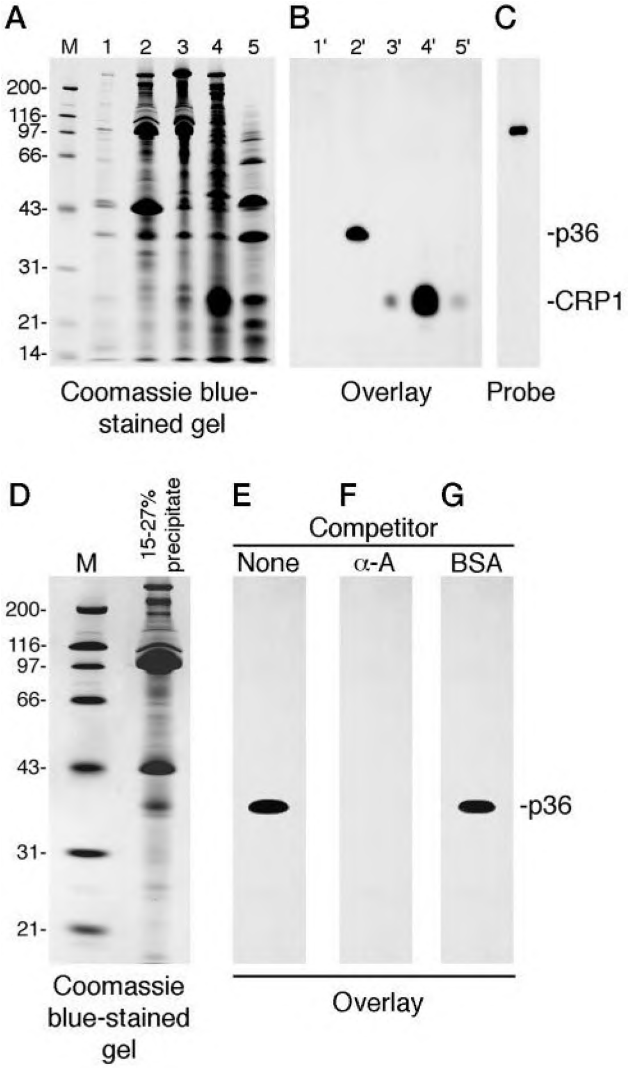

FIG. 1. **A direct and specific interaction between α-actinin and a 36-kDa protein.** *A*, a Coomassie Blue-stained gel showing the molecular mass markers (*M*) and the protein composition of a 0–15% (*lane 1*), a 15–27% (*lane 2*), a 27–34% (*lane 3*), a 34–43% (*lane 4*), and a 43–61% (*lane 5*) ammonium sulfate precipitate from an avian smooth muscle extract. *B*, autoradiograph of a parallel gel transferred to nitrocellulose and probed with [$^{125}$I]α-actinin. The radioiodinated α-actinin binds to CRP1 and to a 36-kDa protein (p36) present in the 15–27% precipitate. *C*, autoradiograph showing the radioiodinated α-actinin probe. *D*, a Coomassie Blue-stained gel showing the molecular mass markers (*M*) and the 15–27% ammonium sulfate precipitate containing p36. Autoradiographs of similar gels transferred to nitrocellulose strips and probed with [$^{125}$I]α-actinin in the absence of competing protein (*E*) or in the presence of a 2,000-fold molar excess of unlabeled α-actinin (*F*) or BSA (*G*). *α-A*, α-actinin.

*Purification and Properties of p36 from Avian Smooth Muscle*—In order to characterize the properties of p36 in greater detail, we developed a method for purifying native protein from smooth muscle. A 15–27% ammonium sulfate precipitate from an avian smooth muscle extract, which is enriched in p36 (see Fig. 1), was used as starting material for the purification of

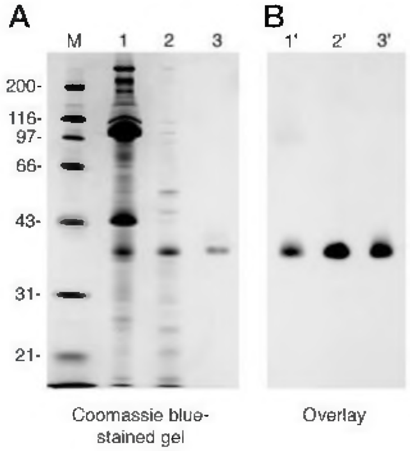

FIG. 2. **Purification of p36 from avian smooth muscle.** *A*, a Coomassie Blue-stained gel showing steps in the purification of p36 from an avian smooth muscle extract: the 15–27% ammonium sulfate precipitate loaded on the DEAE-cellulose column (*lane 1*); proteins that fail to bind the DEAE-cellulose column (*lane 2*); and pooled fractions containing the purified p36 eluted from a CM-cellulose column (*lane 3*). A parallel gel was transferred to nitrocellulose and probed with [$^{125}$I]α-actinin. *B*, the resulting autoradiograph demonstrates that the purified 36-kDa protein is able to bind α-actinin. The position of the molecular mass markers is indicated on the *left* in kDa.

p36. Briefly, proteins present in the 15–27% ammonium sulfate precipitate (Fig. 2*A*, *lane 1*) were applied to a DEAE-cellulose column, and the proteins that fail to bind this matrix (Fig. 2*A*, *lane 2*) were subjected to chromatography on CM-cellulose. The bound proteins were eluted with a linear gradient of NaCl, and the fractions containing p36 were pooled (Fig. 2*A*, *lane 3*). The p36 was purified to apparent homogeneity at this stage. To verify that the 36-kDa protein obtained after these conventional chromatographic techniques was the α-actinin-binding protein we sought, we performed a blot overlay assay using radioiodinated α-actinin. A similar gel to that shown in Fig. 2*A* was transferred to nitrocellulose, and the nitrocellulose was incubated with [$^{125}$I]α-actinin. The autoradiograph revealed that the radioiodinated α-actinin bound to the purified 36-kDa protein. Starting with 400 g of chicken gizzards, this protocol allowed us to obtain 3–4 mg of purified p36.

In order to determine whether p36 exists in a monomeric or multimeric state, we characterized some of the biophysical properties of the purified protein (Table I). The Stokes' radius of the purified avian smooth muscle p36 was estimated by calibrated gel filtration chromatography (28). Three independent experiments revealed a Stokes' radius of 3.1 ± 0.1 nm (mean ± S.E.) for p36 at physiological ionic strength (140 mM NaCl). We have also performed the gel filtration assay in 20 mM NaCl and have obtained similar results (data not shown). A relative sedimentation coefficient of 2.1 ± 0.1 S (mean ± S.E., $n = 3$) was determined for p36 by sucrose density gradient centrifugation (29). The method of Siegel and Monty (23) was employed to estimate a native molecular mass of 25.5 kDa for p36. This value suggests that under our conditions p36 is monomeric. The experimentally determined frictional ratio ($f/f_0$) of 1.6 suggests that p36 is an asymmetric protein.

TABLE I
*Biophysical properties of purified p36*

| | |
|---|---|
| Partial specific volume | 0.711 cm$^3$/g |
| Stokes' radius | 3.1 ± 0.1 nm |
| Relative sedimentation coefficient | 2.1 ± 0.1 S |
| Native molecular mass | 25,500 Da |
| Frictional ratio ($f/f_0$) | 1.6 |

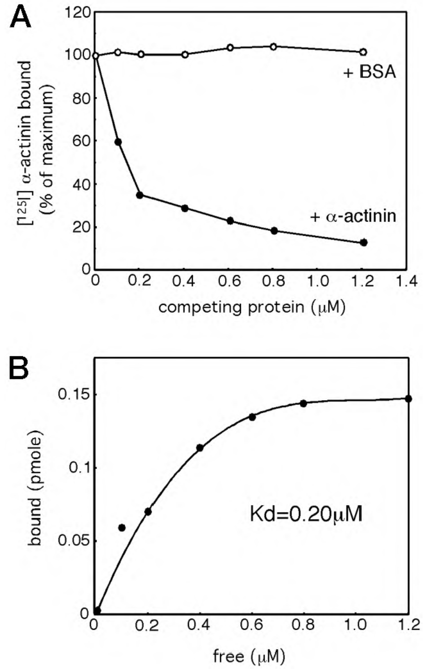

FIG. 3. **Direct and specific interaction between α-actinin and p36 using a solid-phase binding assay.** *A*, microtiter wells were coated with purified p36 and then blocked with BSA. A constant amount of [$^{125}$I]α-actinin was incubated in the wells in the presence of increasing concentrations of unlabeled competing proteins: α-actinin (+ *α-A*) or BSA (+ *BSA*). The amount of bound [$^{125}$I]α-actinin was determined by γ counting. In this particular experiment, the maximal specific binding of the radioiodinated α-actinin to the p36-coated wells in the absence of competing protein corresponds to 3,950 cpm. The data are expressed as a percentage of the maximum counts bound in the absence of competing protein. *B*, from the graph shown in *A*, we have plotted the concentration of bound [$^{125}$I]α-actinin against the concentration of free [$^{125}$I]α-actinin. The calculated dissociation constant ($K_d$) obtained from this experiment was 0.20 μM. From three different experiments, a mean $K_d$ of 0.18 ± 0.04 μM (mean ± S.E.) was calculated.

*A Moderate Affinity Interaction between α-Actinin and p36*—In order to calculate the dissociation constant of the α-actinin-p36 interaction we used a solid-phase binding assay to characterize the interaction under nondenaturing conditions. Briefly, purified p36 was immobilized in microtiter wells and was then exposed to a constant amount of radioiodinated α-actinin in the presence of increasing amounts of unlabeled α-actinin or BSA as competing proteins. The amount of bound [$^{125}$I]α-actinin was determined by γ-counting. As can be seen in Fig. 3*A*, unlabeled α-actinin but not an equivalent molar amount of BSA is able to compete with the radioiodinated α-actinin for binding to p36. From this competition experiment showing the specificity of the interaction under nondenaturing conditions, we were able to plot the moles of bound [$^{125}$I]α-actinin against the moles of free [$^{125}$I]α-actinin (Fig. 3*B*). This curve corresponds to an interaction between two proteins at a single binding site, and in this particular experiment half-maximal binding occurs at 0.20 μM free ligand. An average $K_d$ of 0.18 ± 0.04 μM (mean ± S.E.) corresponding to a moderate affinity interaction between α-actinin and p36 was calculated from three independent experiments.

*Colocalization of α-Actinin and p36 in CEF Cells*—As shown above, we have demonstrated a high specificity, moderate affinity interaction between α-actinin and p36 *in vitro*. If these proteins also interact with each other *in vivo*, we should observe a colocalization of these two proteins in cells. In order to examine this possibility, we have generated polyclonal antibodies against purified smooth muscle p36. As shown in Fig. 4*B*, by Western immunoblot analysis the polyclonal antibodies specifically recognize p36 in a CEF cell lysate (Fig. 4*B*, *lane 1'*), in an avian smooth muscle extract (Fig. 4*B*, *lane 2′*), and after purification (Fig. 4*B*, *lane 3′*). No signal was detected using the preimmune serum under the same conditions (Fig. 4*C*).

We used the specific anti-p36 antibody to compare the subcellular localizations of $\alpha$-actinin and p36 in CEF cells. Double-label indirect immunofluorescence reveals that p36 and $\alpha$-actinin colocalize extensively along the actin stress fibers (Fig. 5, *A–C*), consistent with the view that they might also interact *in vivo*. In order to evaluate if p36 is also present in the focal adhesions at the end of the stress fibers, we performed double label indirect immunofluorescence using anti-p36 antibodies and antibodies directed against vinculin, a well characterized component of focal adhesions. As can be seen in Fig. 5, *D–F*, p36 is found along the actin cytoskeleton as well as in the focal adhesions, where its distribution overlaps with vinculin. Using interference reflection microscopy, we have also observed the localization of p36 in the focal adhesions (data not shown).

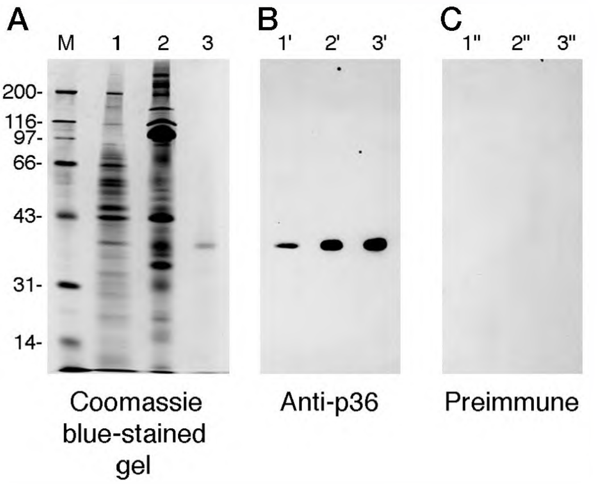

FIG. 4. **Characterization of the p36-antibody.** *A*, Coomassie Blue-stained gel showing the molecular mass markers (*M*), the total proteins from a CEF lysate (*lane 1*), the proteins present in the 15–27% ammonium sulfate precipitate from an avian smooth muscle extract (*lane 2*), and p36 purified from this avian smooth muscle extract (*lane 3*). Corresponding Western immunoblots probed with the polyclonal antibody (K55) raised against p36 (*B*) or the corresponding preimmune serum (*C*) demonstrate the specificity of the p36 antibody. The amount of proteins loaded in *lanes 2′*, *2″*, *3′*, and *3″* was 100 times lower than the amount loaded in *lanes 2* and *3*.

Given the fact that p36 associates with the actin-binding protein $\alpha$-actinin, we examined the possibility that p36 also colocalizes with $\alpha$-actinin in lammellipodia, structures enriched in $\alpha$-actinin and actin, where polymerization of actin occurs during cell spreading. A striking colocalization of $\alpha$-actinin and p36 is observed in the leading lammellipodia of spreading fibroblasts (Fig. 5, *G–I*). No specific staining is observed with the preimmune serum (data not shown). Collectively, these experiments illustrate a striking coincidence in the subcellular distributions of $\alpha$-actinin and p36.

*Evidence for an Interaction between $\alpha$-Actinin and p36 in Vivo*—In an effort to confirm the ability of $\alpha$-actinin to associate with p36 *in vivo*, we performed coimmunoprecipitation experiments in CEF cells using the anti-p36 antibody or the corresponding preimmune serum. The bound material was eluted and resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-p36 or anti-$\alpha$-actinin antibodies. Fig. 6*A* shows that under nondenaturing conditions, p36 can be immunoprecipitated by the anti-p36 antibody but not by the corresponding preimmune serum. Under the same conditions, Western immunoblot analysis of the immunoprecipitated proteins reveals that $\alpha$-actinin is coimmunoprecipitated with p36 (Fig. 6*B*). No signal corresponding to $\alpha$-actinin was detected when the immunoprecipitation was performed using the preimmune serum (Fig. 6*B*). These experiments illustrate that $\alpha$-actinin and p36 are present in the same molecular complex *in vivo* in CEF cells.

*Tissue-specific Expression of p36 Isoforms*—To determine the expression pattern of p36, we performed a Western immunoblot analysis using different tissues derived from 19-day-old chicken embryos. The anti-p36 antibody was used to screen the proteins extracted from brain, heart, arteries, stomach, gizzard, intestine, skeletal muscle, liver, lung, and blood. A Coomassie Blue-stained gel of the protein extracts from each tissue is shown in Fig. 7*A*. A similar gel was transferred to nitrocellulose and probed with the anti-p36 antibody (Fig. 7*B*). p36 is expressed in heart and in tissues enriched in smooth muscle including arteries, stomach, gizzard, intestine, and lung. A second, lower molecular mass immunoreactive band of 33 kDa was detected in heart; at present, it is not clear whether this 33-kDa polypeptide is a proteolytic fragment of p36, is the product of an alternatively spliced transcript, or is a related protein. No signal was detected in brain, liver, or whole blood. Surprisingly, a single protein that exhibits an apparent molec-

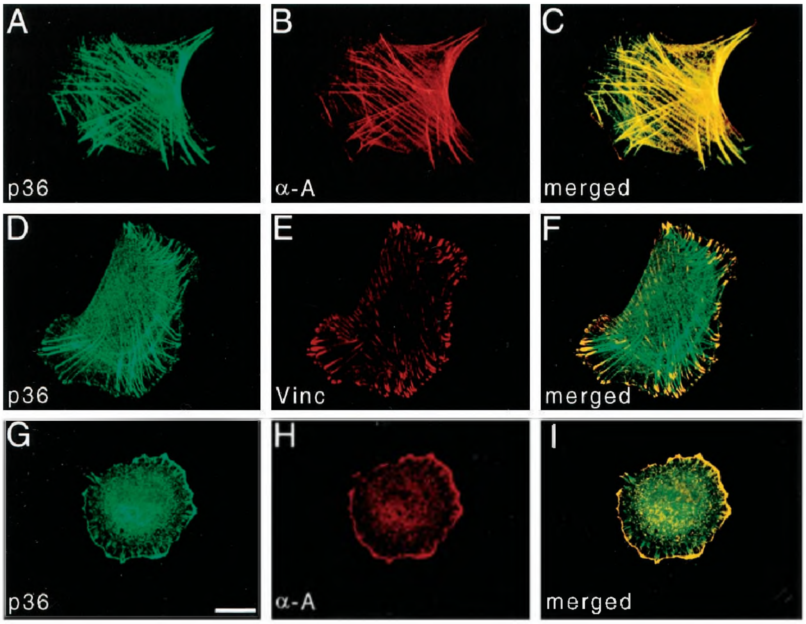

FIG. 5. **Subcellular localization of p36 in CEF cells.** CEF cells were fixed and double-labeled for confocal indirect immunofluorescence microscopy with the polyclonal anti-p36 antibody (*A*, *D*, and *G*), and monoclonal antibodies raised against $\alpha$-actinin (*B* and *H*) or vinculin (*E*). The merged image appears in the *right column* (*C*, *F*, and *I*). $\alpha$-Actinin and p36 are colocalized along the actin stress fibers of well spread cells (*C*) and in the lammellipodia of cells that have been plated on fibronectin for 15 min (*I*). The p36 staining also overlaps with the vinculin staining at the end of the actin stress fibers in the focal adhesions (*F*). *Bar*, 30 $\mu$M.

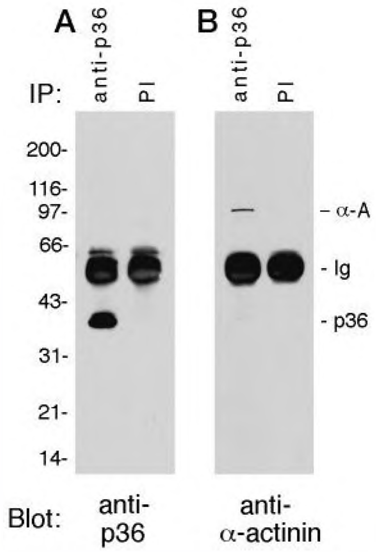

FIG. 6. **Coimmunoprecipitation of α-actinin with p36 in CEF cells.** Proteins were immunoprecipitated from a CEF lysate in radioimmune precipitation buffer with the anti-p36 antibody (anti-p36) or the corresponding preimmune serum (*PI*). Immunoprecipitated proteins were revealed by Western immunoblotting using the anti-p36 antibody (*A*) or a polyclonal antibody raised against α-actinin (*B*). Western immunoblot analysis shows that α-actinin is immunoprecipitated with p36 under nondenaturing conditions when the anti-p36 antibody, but not the preimmune serum, is used. The positions of the molecular mass markers are indicated in kDa as well as the positions of α-actinin (*α-A*), p36 (*p36*), and the IgG heavy chain (*Ig*).

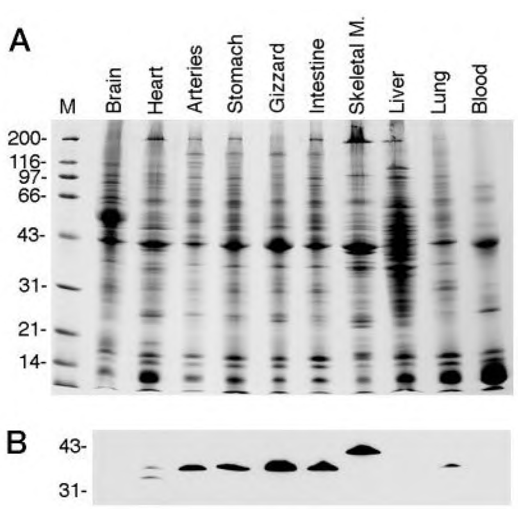

FIG. 7. **Tissue distribution of p36.** *A*, a Coomassie Blue-stained gel showing the proteins extracted from different tissues derived from 19-day-old chicken embryos. *B*, corresponding Western immunoblot probed with the anti-p36 antibody reveals that p36 is expressed in heart and in tissues enriched in smooth muscle (arteries, stomach, gizzard, intestine, and lung), whereas a 40-kDa isoform of p36 is found in skeletal muscle. The position of the molecular mass markers is indicated on the *left* in kDa.

ular mass of 40 kDa (p40) was prominent in skeletal muscle, suggesting the presence of a larger isoform in skeletal muscle cells. No proteins were detected when the preimmune serum was used in this Western immunoblot analysis (data not shown). These immunoblot results revealed the existence of at least two immunologically related proteins that display distinct patterns of muscle-specific expression.

*Up-regulation of p36 and p40 Expression during Myogenic Differentiation*—In order to examine a possible role of p36 during myogenesis, we evaluated the expression level of p36 in arteries, a tissue enriched in smooth muscle, during embryogenesis (Fig. 8*A*). The level of p36 in arteries increases dramatically as a function of developmental time between day 11 and day 15 (Fig. 8*B*). A similar result but with a less striking increase was also observed during the development of another smooth muscle-rich organ, the gizzard, from 11–18-day-old chicken embryos (data not shown).

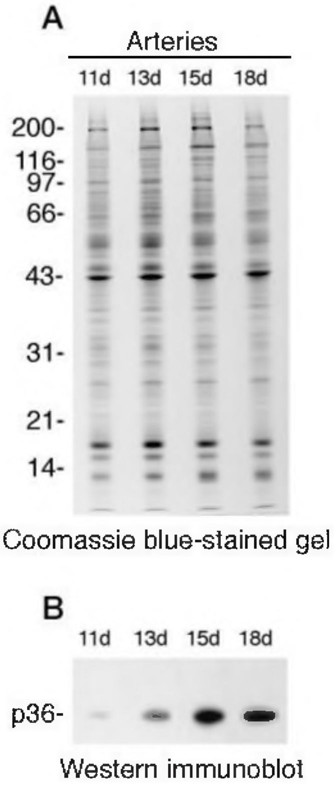

FIG. 8. **Up-regulation of p36 expression in differentiated smooth muscle cells.** *A*, Coomassie Blue-stained gel showing the proteins extracted from arteries derived from 11-day (*11d*), 13-day (*13d*), 15-day (*15d*), and 18-day old (*18d*) chicken embryos. *B*, a parallel gel was transferred to nitrocellulose and analyzed by Western immunoblotting using the anti-p36 antibody. The expression level of p36 in arteries increases markedly during development. The position of the molecular mass markers is indicated on the *left* in kDa.

In parallel studies, we used the myogenic C2C12 cell line to examine the expression of p40 during striated muscle development (Fig. 9). C2C12 myoblasts proliferate in the presence of high serum and are induced to differentiate upon removal of growth factors. Proteins present in undifferentiated and differentiated C2C12 cells were resolved by SDS-PAGE (Fig. 9*A*). By Western immunoblot analysis, no p40 is detected in undifferentiated myoblasts, but p40 expression is induced upon differentiation (Fig. 9*B*). An immunoreactive polypeptide with an apparent molecular mass of 35 kDa is also detected in the differentiated C2C12 lysate. The significance of this band is unknown, but it was not detected by Western immunoblot in the skeletal muscle extract from chicken embryo shown in Fig. 7. The up-regulated expression of p36 and p40 in smooth and skeletal muscle is consistent with the possibility that these proteins have specialized roles within differentiated muscle. The subcellular distribution of p40 in differentiated C2C12 myotubes was evaluated by double label indirect immunofluorescence and compared with the distribution of α-actinin. As can be seen in Fig. 9, *C–E*, p40 colocalizes precisely with α-actinin at the Z-lines of the myotubes.

*Molecular Cloning of cDNAs Encoding Chicken p36 and p40*—The relationship between the p36 and the p40 proteins

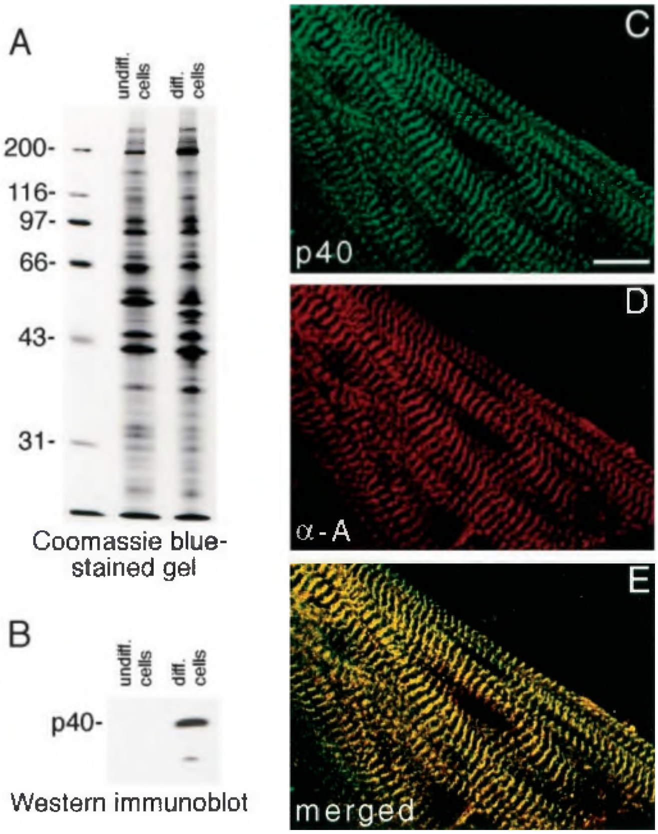

FIG. 9. **Up-regulation of p40 expression in differentiated C2C12 myotubes.** *A*, Coomassie Blue-stained gel showing the total proteins from undifferentiated C2C12 cells (*undiff. cells*) and differentiated C2C12 cells (*diff. cells*). The positions of the molecular mass markers are indicated on the left in kDa. *B*, Western immunoblot analysis of the proteins extracted from the C2C12 myogenic cell line using the K55 antibody raised against p36. Under proliferation conditions no p40 is detected in the lysate of undifferentiated myoblasts (*undiff. cells*), whereas under differentiation conditions p40 expression is induced in myotubes (*diff. cells*). Differentiated C2C12 cells were double-labeled using the polyclonal K55 antibody raised against p36 (*C*) and a monoclonal antibody raised against sarcomeric α-actinin (*D*). p40 staining (*green*) and α-actinin staining (*red*) are also shown after merging (*E*). Overlapping regions appearing in *yellow* reveal that p40 colocalizes with α-actinin at the Z-lines of the differentiated myotubes. *Bar*, 20 $\mu$m.

was defined by analysis of cDNAs that encode the two proteins (Fig. 10). In an effort to isolate cDNAs encoding p36, we obtained peptide sequence from the purified protein. The N terminus as well as several peptides derived by endoproteolytic cleavage were sequenced. By this approach, we obtained 147 aa of peptide sequence, an estimated 47% of the total protein sequence. Degenerate primers were designed based on these p36-derived sequences. These primers were used to amplify a fragment of 296 bp from first strand CEF cDNA; the deduced amino acid sequence of the resulting product extended bona fide p36 sequence beyond what was encoded by the primers, confirming the relationship of the polymerase chain reaction product to a p36 transcript. Based on the nucleotide sequence of this fragment, two internal primers were synthesized and used to generate a transcript-specific nucleotide probe. One million recombinants from a total chicken embryo cDNA library were screened with the p36 probe. Five positive plaques were isolated and purified. Two of the five isolates, clone 1 and clone 8, were sequenced in entirety on both strands.

The clone 1 cDNA insert is 1280 bp in length. The nucleotide sequence reveals a potential initiation codon (ATG) at nucleotides 47–49, and a stop codon (TAG) at nucleotides 992–994. A Kozak consensus initiation sequence (GCCACG) is present just before the initiation codon (30), and a polyadenylation signal (AATAAA) is found at the 3′-end of the cDNA (31). An open reading frame of 945 bp encodes a protein of 315 amino acids with a predicted molecular mass of 34.2 kDa. Analysis of the protein sequence shown in Fig. 10*A* reveals the presence of two protein-binding motifs: a PDZ domain at the N terminus and a LIM domain at the C terminus. All eight peptide sequences obtained by microsequence analysis of the purified p36 are found in the deduced 315-amino acid protein sequence (Fig. 10*A*), demonstrating unambiguously that the cDNA we have cloned encodes p36.

We also isolated another cDNA insert from clone 8 that encodes a partial protein product of 298 aa with a high degree of identity to p36. The nucleotide sequence and deduced amino acid sequence are essentially identical to that of clone 1 except in the central region, where 189 nucleotides (encoding 63 aa) of clone 1 are replaced by 333 nucleotides (encoding 111 aa) of unique sequence in clone 8 (Fig. 10*B*). This change is sufficient to account for the molecular weight difference between p36 and p40. Thus, we believe that we have cloned a cDNA encoding the skeletal muscle p40 isoform. Further, it appears that transcripts encoding p36 and p40 are derived by alternative splicing of the same precursor RNA.

Although the nucleotide sequence of p36 cDNA is nearly identical to that of p40 cDNA, except in the central unique region described above, we did observe four examples of nucleotide substitutions when comparing the common regions of p36 and p40 cDNAs. There are two nucleotide substitutions that affect the amino acid sequence (Fig. 10*A*). The GAG codon at nucleotides 287–289 coding for a glutamic acid at position 81 in p36 is replaced by the AAG codon coding for a lysine in p40. A second substitution replaces the GCG codon at nucleotides 578–580 coding for an alanine at position 178 in p36 by the ACG codon coding for a threonine at position 226 in p40. As will be discussed further below, these changes could reflect polymerase errors that occurred during the production of the library or they could represent RNA editing events mediated by adenosine deamination (32). In addition, we observed two substitutions (a cytosine at position 268 and a thymine at position 604 in the clone encoding p36 are replaced by a thymine and a cytosine, respectively, in the cDNA encoding p40) that are probably due to polymorphisms at the locus.

Analysis of the predicted amino acid sequences of avian p36 and p40 revealed a high degree of similarity to recently described rat and human muscle proteins called the ALPs (1). Thus, we believe that the proteins we have described here represent the 36- and 40-kDa avian isoforms of ALP. We suggest calling the p36-ALP isoform smALP (smooth muscle ALP), based on its prevalence in smooth muscle, and the p40-ALP isoform skALP (skeletal muscle ALP), based on its presence in skeletal muscle.

## DISCUSSION

In this paper, we describe the identification, purification, and characterization of avian smALP, a cardiac and smooth muscle protein that interacts with the actin-binding protein, α-actinin. We have developed a method for purifying smALP from avian smooth muscle and have characterized its biophysical properties. A variety of binding assays were employed to demonstrate a direct, specific, and saturable interaction between smALP and α-actinin. SmALP and α-actinin display a moderate affinity interaction *in vitro* with an average calculated $K_d$ of 0.18 $\mu$M. Moreover, we have used native immunoprecipitation to demonstrate that smALP and α-actinin are present in the same molecular complex *in vivo*. Further support for the *in vivo* relevance of the smALP-α-actinin interaction comes from their co-localization within cells.

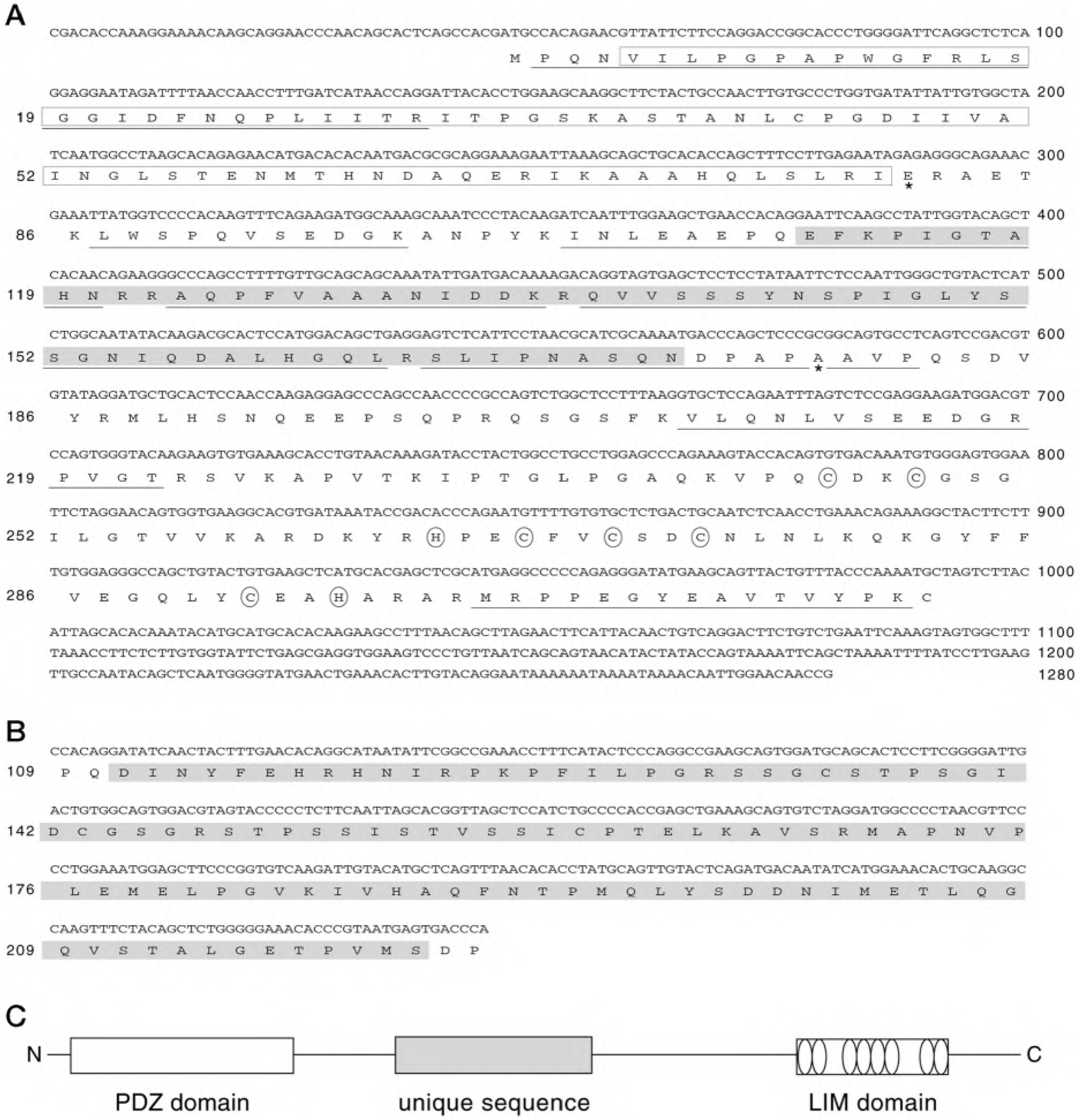

FIG. 10. **Sequence analysis of chicken p36 and p40 cDNAs.** *A*, the nucleotide sequence (*numbered* on the *right*) of clone 1 and the deduced amino acid sequence (*numbered* on the *left*) corresponding to p36 are shown. The amino acid sequence corresponding to the PDZ domain is *boxed*, whereas cysteine and histidine residues contributing to the LIM motif are *circled*. The unique amino acid sequence of p36 is *shaded*. The peptide sequences obtained by microsequencing of p36 are *underlined*. An *asterisk* indicates the two amino acids present in p36 (at positions 81 and 178) that are different amino acids in p40 based on the nucleotide sequences of clone 1 and clone 8, respectively (see "Results" for details). *B*, the unique amino acid sequence of p40 is *shaded*. *C*, model showing the domain structure of p36.

In the course of these studies, we also identified skALP, a 40-kDa protein that is closely related to smALP and that is expressed exclusively in skeletal muscle. Thus, all three vertebrate muscle types exhibit expression of an ALP isoform. Other than fibroblasts, which typically express a protein repertoire reminiscent of smooth muscle, muscle cells appear to be the primary site of ALP expression in the chick. The fact that neither smALP nor skALP appear to be expressed substantially in nonmuscle derivatives suggests that their physiological role may be related to some differentiated function of muscle. In further support of this notion, we observed that ALP expression levels increase during smooth muscle and skeletal muscle differentiation. Moreover, skALP is localized to the Z-line of differentiated myotubes, suggesting a role for ALP isoforms in muscle organization and/or function.

The rat skeletal muscle form of ALP was described recently by Xia *et al.* (1), who identified the protein in the course of a search for PDZ domain proteins in skeletal muscle and described an interaction between ALP and α-actinin using a two-hybrid screen. Of particular interest, Xia and colleagues performed chromosomal mapping studies to show that the gene encoding ALP maps to human chromosome 4q35 (1), close to a region of heterochromatin that is deleted in individuals afflicted with facioscapulohumeral muscular dystrophy, the third most common form of inherited muscle disease (33). It has been postulated that the heterochromatin deletion alters the expression of some nearby gene that is essential for some aspect of muscle function (34). Thus, the *ALP* gene has emerged as a candidate for the gene affected in facioscapulohumeral muscular dystrophy. As might be expected for a protein that plays an important role in muscle function, Xia *et al.* (1) showed that rat skALP displays dramatically up-regulated expression during skeletal muscle differentiation. Our work on the avian ALPs confirms and extends the findings of Xia *et al.* by presenting a method for isolation of native ALP from muscle, the characterization of its biophysical properties and association with α-actinin, and the demonstration of a smooth muscle isoform of ALP. The availability of purified ALP will allow detailed analysis of its biochemical role in muscle.

Molecular cloning and analysis of the cDNAs encoding chicken smALP and skALP has confirmed their relationship to each other. The N-terminal and C-terminal regions of the proteins are identical in sequence. However, the two isoforms differ in an internal sequence; 63 aa present in smALP are replaced by a unique sequence of 111 aa in skALP. Based on the absolute identity of the cDNA sequences outside this central region, it appears that p36 and p40 are encoded by transcripts derived by alternative splicing. We speculate that the unique central domains in the ALP isoforms confer some functional specificity, perhaps reflecting the distinct properties of the different muscle subtypes in which they are exclusively expressed. Additional work will be required to define the physiological relevance of these novel domains.

In addition to the differences in the central regions of smALP and skALP, we also observed two nucleotide substitutions that are predicted to affect the amino acid sequence of the proteins, changing the glutamate codon (GAG) at position 81 in smALP to lysine (AAG) and the alanine codon (GCG) at position 178 in smALP to threonine (ACG). In both cases, we observe an A in the skeletal muscle skALP cDNA and a G in the smALP cDNA. It is possible that these differences occurred during the production of the cDNA library and have no physiological significance or that they reflect polymorphisms. Alternatively, these differences could be the result of RNA editing. RNA-specific adenosine deaminases convert adenosine to inosine, which would result in an A to G nucleotide change in the coding strand of a cDNA and can thus modulate protein structure and function (32). RNA-specific adenosine deaminases are present at very low levels in skeletal muscle (35), consistent with the observation that the skALP cDNA that is derived from a skeletal muscle transcript exhibits an adenine nucleotide. Also, the positions of the nucleotide differences are near an apparent intron-exon boundary, as commonly occurs for RNA-specific adenosine deaminase-dependent changes, since double-stranded RNA is required for the activity (32). The peptide sequence obtained by microsequencing of smALP (aa 165–181) shows that the amino acid at position 178 in our preparation of smALP is a threonine; thus, if site-specific deamination did occur, it must not be complete or could be developmentally regulated. Genomic sequencing will be required to establish whether the difference reflected at the level of the cDNAs has physiological relevance.

The domain structures of both smALP and skALP suggest their ability to dock multiple protein partners. The two proteins each display an identical N-terminal PDZ domain and C-terminal LIM domain. PDZ domains are 80–100-amino acid motifs that mediate specific protein-protein interaction (16). LIM motifs are cysteine-rich domains approximately 60 amino acids in length (36) that coordinate two zinc atoms (37) and also serve as protein binding interfaces (17). The presence of these two protein binding domains in smALP and skALP suggests that the proteins could act as a linker or adaptor molecules within the contractile machinery of muscle cells. Insight into the nature of the α-actinin binding site of ALP has recently emerged; domain analysis revealed that it is the PDZ domain of skALP that interacts with α-actinin (1). Because LIM domains also represent protein binding interfaces, it will be of importance in the future to determine what protein partner or partners associate with the LIM domain present in ALP.

The findings that ALP isoforms are expressed specifically in muscle cells, are up-regulated during muscle differentiation, and are associated with α-actinin at key sites for muscle cytoarchitecture raise the possibility that these PDZ-LIM proteins may cooperate with α-actinin to stabilize and/or strengthen the contractile machinery of muscle cells. Given this hypothesis and the human genetic mapping data that suggest the *ALP* gene as a candidate for the gene that is critically affected in facioscapulohumeral muscular dystrophy (1), it will be of particular interest to assess the involvement of ALP in facioscapulohumeral muscular dystrophy and to define its role in muscle structure and function.

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