Analysis of the Actin-binding Domain of α-Actinin by Mutagenesis and Demonstration That Dystrophin Contains a Functionally Homologous Domain

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Abstract. To define the actin-binding site within the NH_2-terminal domain (residues 1–245) of chick smooth muscle α-actinin, we expressed a series of α-actinin deletion mutants in monkey Cos cells. Mutant α-actinins in which residues 2–19, 217–242, and 196–242 were deleted still retained the ability to target to actin filaments and filament ends, suggesting that the actin-binding site is located within residues 20–195. When a truncated α-actinin (residues 1–290) was expressed in Cos cells, the protein localized exclusively to filament ends. This activity was retained by a deletion mutant lacking residues 196–242, confirming that these are not essential for actin binding. The actin-binding site in α-actinin was further defined by expressing both wild-type and mutant actin-binding domains as fusion proteins in *E. coli*. Analysis of the ability of such proteins to bind to F-actin in vitro showed that the binding site was located between residues 108 and 189. Using both in vivo and in vitro assays, we have also shown that the sequence KTFT, which is conserved in several members of the α-actinin family of actin-binding proteins (residues 36–39 in the chick smooth muscle protein) is not essential for actin binding. Finally, we have established that the NH_2-terminal domain of dystrophin is functionally as well as structurally homologous to that in α-actinin. Thus, a chimeric protein containing the NH_2-terminal region of dystrophin (residues 1–233) fused to α-actinin residues 244–888 localized to actin-containing structures when expressed in Cos cells. Furthermore, an *E. coli*–expressed fusion protein containing dystrophin residues 1–233 was able to bind to F-actin in vitro.

α-ACTININ is a rod-shaped F-actin cross-linking protein found in both muscle and nonmuscle cells at sites where actin is attached to a variety of intracellular structures. It is a homodimer with subunits of molecular mass 94–103 kD arranged in an antiparallel orientation. Smooth, skeletal, and nonmuscle isoforms of the protein have been identified, the only clear functional difference being that binding of the nonmuscle isoform to actin is inhibited by calcium, whereas the muscle isoforms are calcium insensitive in this respect (reviewed in Blanchard et al., 1989). Analysis of the deduced sequence of the chick smooth muscle isoform of the protein shows that α-actinin can be divided into three distinct domains, an NH_2-terminal actin-binding domain spanning approximately the first 245 amino acid residues, four spectrin-like repeats (residues 246–713), and a COOH-terminal region (residues 714–888) containing two EF-hand calcium binding motifs (Baron et al., 1987). Sequence comparisons show that the actin-binding domain of α-actinin is homologous with that found in a number of other actin-binding proteins including β-spectrin (Karinch et al., 1990), fimbrin (de Arruda et al., 1990), filamin (Gorlin et al., 1990), and the *Dictyostelium discoideum* 120-kD actin gelation factor called ABP-120 (Noegel et al., 1989). The NH_2-terminal region of dystrophin also shows sequence similarity to the actin-binding domains found in this family of proteins, suggesting that dystrophin is also likely to be an actin-binding protein (Hammonds, 1987; Davison and Critchley, 1988). However, at present there is no direct evidence that dystrophin can bind actin.

The actin-binding domain of α-actinin can be liberated from the whole molecule as a 27-kD polypeptide by thermolysin digestion, and this polypeptide retains the ability to bind to F-actin (Mimura and Asano, 1986). However, little is known about those sequences within this domain which are essential for actin binding. Studies on the actin-binding domains of other members of this family of actin-binding proteins have begun to provide some information in this re-

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Since this work was submitted for publication, Bresnick et al. (Bresnick, A. R., P. A. Janmey, and J. Condeelis. 1991. *J. Biol. Chem.* 268:12989–12993) have shown that antibodies to the 27 amino acids implicated in binding of ABP120 to actin can immune precipitate ABP120 from cell lysates, suggesting that these residues are on the surface of the protein as expected for an actin-binding site. Fab' fragments of the antibody inhibited ABP120 binding to actin, and the synthetic 27 mer inhibited actin cross-linking by ABP120. Evidence was also presented that the synthetic 27 mer cosediments with actin.

gard. Thus, a human β-spectrin tryptic fragment (16.5 kD) spanning residues 47 through to approximately 186 has been shown to possess F-actin-binding activity (Karinch et al., 1990). Similarly, tryptic digestion of ABP-120 yields a 17-kD polypeptide which is reported to bind F-actin, whereas a 14-kD tryptic fragment derived from this polypeptide by loss of a further 27 NH_2-terminal residues was unable to do so. These 27 amino acids span residues 89–115 in the sequence of ABP-120 (Bresnick et al., 1990). Levine et al. (1990) have also reported that a synthetic peptide based on dystrophin residues 10–32 was able to interact with F-actin as judged by nuclear magnetic resonance. This region contains the sequence KTFT which is found in the actin-binding domain of several of this family of actin-binding proteins.

We have previously shown that when a cDNA encoding the chick smooth muscle isoform of α-actinin was expressed in monkey Cos cells, the expressed protein targeted to actin microfilaments and to filament ends where they terminate at cell-matrix junctions (Jackson et al., 1989). This distribution mirrors that of the endogenous α-actinin, and is thought to reflect the ability of the protein to bind to F-actin. In this study we have used mutagenesis in conjunction with this assay to further define the actin-binding site within α-actinin. We have also expressed the actin-binding domain of α-actinin in *Escherichia coli* as a fusion protein with glutathione-*s*-transferase (GST)[1] and have monitored the effects of deletions and point mutations on the ability of the fusion protein to bind to F-actin in vitro. The results of these studies show that the actin-binding site in α-actinin is located between residues 108 and 189. Finally, we have tested the hypothesis that dystrophin contains a functional actin-binding site. Thus, we have replaced the actin-binding domain of α-actinin with the equivalent domain from dystrophin and investigated the ability of the expressed protein to target to actin-containing structures in Cos cells. The NH_2-terminal domain of dystrophin has also been expressed as a fusion protein in *E. coli* and its ability to bind to F-actin assayed in vitro. The results clearly demonstrate that dystrophin contains a functional actin-binding site.

Materials and Methods

Plasmid Constructs Used in Transient Expression Experiments

A full-length chick smooth muscle α-actinin cDNA (clone C17) (Baron et al., 1987) was isolated as an EcoRI restriction enzyme fragment from a Bluescript construct. The ends were filled in and the fragment blunt-end ligated into the SmaI site of the eukaryotic plasmid expression vector pSVL40, which contains the SV40 late promoter, poly(A) addition sequence and poly(A) tract (Pharmacia Fine Chemicals, Uppsala, Sweden). This construct is referred to as pSVL/C17 (Fig. 1). Deletion of the nucleotides encoding residues 1-218 was achieved by purifying a 3′ EcoRV-EcoRI restriction enzyme fragment from C17 (Fig. 1), followed by blunt-end ligation into the SmaI site of pSVL40. Translation from this construct depends on initiation at methionine 222. This mutant is referred to as pSVL/N222 (Fig. 1). The authentic initiating methionine codon is retained in all remaining constructs. Other deletion mutants as well as point mutations were made by site-directed mutagenesis using C17 subcloned into the EcoRI site of the M13-based vector, mICE mp18 (Eperon, 1986). The frequency of isolation of mutants was increased by preparing single-stranded template in the dut^- ung^- strain of *E. coli* (Kunkel et al., 1987). Screening for mutants was carried out by T-track sequencing using the dideoxy chain termination method of Sanger et al. (1977). All mutants were sequenced across the region of interest before subcloning into pSVL40.

A 5′ EcoRI-BamHI restriction enzyme fragment of C17 which encodes the NH_2-terminal actin-binding domain of α-actinin (approximately residues 1-245) and terminates at residue 290 (Fig. 1) was blunt-end ligated into the SmaI site of pSVL40 (pSVL/ABD). A similar construct (Fig. 1) in which the nucleotides encoding residues 196-242 had been deleted by mutagenesis was generated from a mutant designated pSVL/C195 by excision of a 1.8-kb 3′ BamHI fragment (BamHI cuts once in C17 and once in the pSVL40 polylinker 3′ to the SmaI site). The cut plasmid was gel-purified and religated (pSVL/ABD195).

A pSVL40 construct encoding NH_2-terminal residues 1-233 of human dystrophin fused to residues 244–888 of chick smooth muscle α-actinin, and termed pSVL/DαA (Fig. 1), was generated as follows. An XbaI site was

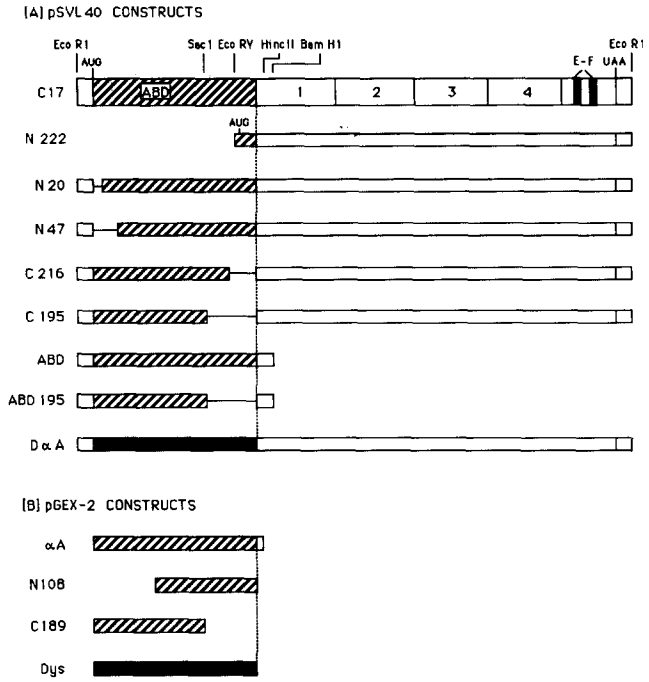

Figure 1. α-Actinin and dystrophin cDNA constructs. C17 is a 3.6-kb cDNA encoding the complete 888 residues of chick smooth muscle α-actinin (Baron et al., 1987). The approximate position of useful restriction enzyme sites as well as the domain structure of the protein are shown. The actin-binding domain (*ABD*) of α-actinin spanning residues 1-245 (numbering includes initiating methionine) is indicated by shading. The four spectrin-like repeats found in α-actinin (residues 246–713) are numbered, and the approximate position of the two EF-hand calcium binding motifs (*E–F*) is also shown. The following α-actinin constructs were made in the eukaryotic expression vector pSVL40 (*A*): pSVL/C17 encodes full-length chick α-actinin; pSVL/N222, deletion of residues 1-221; pSVL/N20, deletion of residues 2-19; pSVL/N47, deletion of residues 2-46; pSVL/C216, deletion of residues 217-242; pSVL/C195, deletion of residues 196-242. The pSVL/ABD construct was derived from pSVL/C17 and encodes the actin-binding domain of α-actinin extending up to residue 290; pSVL/ABD195 was derived from pSVL/C195; pSVL/DαA encodes residues 1-233 of human dystrophin (shown in black) fused to residues 244–888 of chick α-actinin. The authentic AUG translation start codon was retained in all pSVL40 constructs except for pSVL/N222, which relies on initiation at an internal AUG encoding methionine 222. α-Actinin and dystrophin cDNAs were also expressed as GST fusion proteins using the prokaryotic vector pGEX-2 (*B*). The constructs were: pGEX/αA, encodes α-actinin residues 1-269; pGEX/N108, encodes α-actinin residues 108–242; pGEX/C189, encodes α-actinin residues 1-189; and pGEX/Dys, encodes dystrophin residues 1-233.

1. *Abbreviations used in this paper*: GST, glutathione-*s*-transferase; PCR, polymerase chain reaction.

introduced into the α-actinin clone C17 (using site-directed mutagenesis) at the junction between residues 244 and 245. This was achieved by a single base change which resulted in the substitution of an arginine for serine at residue 245. This construct was excised from the mICE vector as an EcoRI fragment, and blunt-end ligated into the SmaI site of pSVL40. Nucleotides encoding residues 1–243 were excised from the recombinant plasmid using a 5′ XhoI site within the pSVL40 polylinker and the newly introduced XbaI site within C17. This fragment was replaced by a polymerase chain reaction (PCR) fragment (with compatible restriction sites) encoding residues 1–233 of human dystrophin. The human dystrophin plasmid construct pCF27 (Koenig et al., 1987) (kindly donated by Dr. D. Love, John Radcliffe Hospital, Oxford, UK) was used as template for the PCR reaction. pCF27 consisted of pUC13 containing a 2.2-kb cDNA insert corresponding to the 5′ end of the human dystrophin molecule. The pSVL/DαA construct was authenticated both by restriction mapping and DNA sequencing.

Transient Expression of pSVL40 Plasmid Constructs in Monkey Cos Cells

Plasmid DNA for transfection was prepared using the alkaline lysis method followed by purification using polyethylene glycol precipitation as described by Sambrook et al. (1989). The sequence of all constructs was confirmed by double-stranded sequencing (Mierendorf and Pfeffer, 1987) of plasmid DNA prepared for transfection. Transfection of plasmid DNA into Cos cells was carried out using the DEAE-dextran method (Cullen, 1987) as previously described (Jackson et al., 1989) using 50 μg of DNA per 100-mm dish. For immunofluorescence localization of the expressed α-actinin, cells (40 h after transfection) were permeabilized with 0.5% (vol/vol) Triton X-100 in 50 mM MES buffer, pH 6.0, 3 mM EGTA, and 5 mM $MgCl_2$ for 2 min and fixed with 3.7% (wt/vol) formaldehyde in PBS (15 min). The expressed chick α-actinin was detected exactly as described previously (Jackson et al., 1989) using a rabbit antibody against chicken gizzard α-actinin which had been preadsorbed against permeabilized monkey Cos cells. The antibody, which is specific for chick α-actinin (Jackson et al., 1989) has been shown to recognize epitopes within the actin-binding domain and spectrin-like repeats of α-actinin (data not shown). For immunofluorescence, bound antibody was detected using a Texas red-labeled anti-rabbit antibody (Amersham International, Amersham, UK). Both antibodies were diluted 1:50 in PBS before use. Cells were costained for F-actin using NBD-phallacidin (Molecular Probes, Inc., Eugene, OR). Metabolic labeling of cell cultures with [^{35}S]methionine, immune precipitation, and detection of labeled proteins were carried out as described previously (Jackson et al., 1989).

Expression of the Actin-binding Domains of α-Actinin and Dystrophin as GST Fusion Proteins in E. coli

An NcoI-HincII DNA restriction fragment derived from the smooth muscle α-actinin cDNA C17 and encoding the actin-binding domain together with an additional 27 COOH-terminal amino acids (residues 1–269) was initially subcloned into the NcoI/StuI sites of the pET3 expression vector (a construct kindly donated by Dr M. Wey, Medical Research Council Molecular Biology Laboratories, Cambridge, UK). This fragment was excised from pET3 using flanking BamHI and EcoRI restriction sites and cloned in frame into the GST gene contained in the prokaryotic plasmid expression vector pGEX-2 (Pharmacia Fine Chemicals). The resulting construct was designated pGEX/αA (Fig. 1). A construct encoding residues 108–242 of chick smooth muscle α-actinin fused in frame to GST (pGEX/N108) (Fig. 1) was generated by PCR using the cDNA clone C17 as template. The 5′ oligonucleotide primer contained a BamHI site and the 3′ primer an EcoRI site, enabling the PCR product to be force-cloned into BamHI/EcoRI cut pGEX-2. A construct encoding residues 1–189 of chick smooth muscle α-actinin fused to GST (pGEX/C189) (Fig. 1) was made by cutting the pGEX/αA construct at a convenient SacI site within the actin-binding domain and at the EcoRI site within the pGEX-2 polylinker. After removal of the SacI-EcoRI fragment, the vector was religated after the ends were filled in using T4 DNA polymerase 1. Constructs encoding residues 2–242 of α-actinin, but in which the KTFT sequence had been mutated, were generated by PCR using 5′ and 3′ primers containing BamHI and EcoRI sites, respectively, and the pSVL/KTFT mutant constructs as templates. PCR products were force-cloned into BamHI/EcoRI cut pGEX-2. A DNA fragment encoding residues 1–233 of human dystrophin was synthesized by PCR and cloned in frame into the SmaI site of pGEX-2 (pGEX/Dys) (Fig. 1). The validity of all constructs was established by restriction enzyme analysis and double-strand sequencing. The GST fusion proteins were expressed in *E. coli* and purified from cell lysates exactly as described by Smith and Johnson (1988) using a glutathione-agarose affinity matrix (Sigma Chemical Co., St. Louis, MO).

Actin Cosedimentation Assay

The ability of the GST fusion proteins to bind actin was investigated using an actin cosedimentation assay. 5 μl of rabbit muscle G-actin (5 μg/μl; Sigma Chemical Co.) and the appropriate fusion protein were mixed in a total volume of 100 μl of buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM dithiothreitol, 1 mM NaN_3, 0.2 mM $CaCl_2$). The fusion proteins under analysis were relatively insoluble in this buffer and Triton X-100 (0.1% vol/vol) was therefore added to reduce sedimentation of the fusion proteins in the absence of actin. Actin polymerization was initiated by the addition of 3 mM $MgCl_2$ and 100 mM NaCl, and the samples were incubated for 1 h at room temperature. Samples were centrifuged with or without actin in an airfuge (Beckman Instruments, Inc., Fullerton, CA) at 85,000 rpm (100,000 *g*) for 30 min. The entire supernatant and pellet were analyzed by SDS-PAGE (12% acrylamide). Where the size of the fusion protein tested differed substantially from that of actin, gels were simply stained with Coomassie blue. Unfortunately, fusion proteins encoded by pGEX/N108 and pGEX/C189 (41 and 48 kD, respectively) were difficult to resolve from the actin (43 kD). To overcome this problem, proteins resolved by SDS-PAGE were transferred to nitrocellulose and the α-actinin fusion proteins detected with antibodies to α-actinin. The pGEX/N108 fusion protein (α-actinin residues 108–242) was detected using the polyclonal rabbit anti-chicken gizzard α-actinin antibody. The pGEX/C189 fusion protein (α-actinin residues 1–189) was not detected using this antiserum, and a monoclonal antibody to human platelet α-actinin (a generous gift from Dr. J. M. Wilkinson, Royal College of Surgeons, London, UK) was used in this case. Bound antibodies were revealed using 1:10,000 dilutions of alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG's (Promega Corp., Madison, WI), respectively.

Results

Expression of Chick α-Actinin in Monkey Cos Cells

A cDNA (C17) encoding the complete sequence of chick smooth muscle α-actinin (Baron et al., 1987) was subcloned into the eukaryotic expression vector pSVL40 (pSVL/C17). The chick α-actinin was transiently expressed in monkey Cos cells and the intracellular distribution of the expressed protein was detected by immunofluorescence using an antibody rendered specific for chick α-actinin. The expressed protein was distributed along actin microfilaments, and was also localized to filament ends where they terminate at cell-matrix junctions (adhesion plaques) (Fig. 2, *A* and *B*).

To establish that targeting of chick α-actinin to these structures was entirely due to the actin-binding domain of the protein, a plasmid construct (pSVL/N222) was made in which the nucleotides encoding NH_2-terminal residues 1–218 were deleted by excision of a 5′ EcoRI-EcoRV restriction enzyme fragment (Fig. 1). Expression of the truncated protein (molecular mass 70 kD), which is dependent on translation initiating at methionine residue 222, was confirmed by immune precipitation using lysates from transfected Cos cells grown in the presence of [^{35}S]methionine (data not shown). Immunofluorescence studies showed that the mutant α-actinin was unable to localize to either actin filaments or adhesion plaques, although the integrity of these structures was confirmed by counterstaining for actin using NBD-phallicidin (Fig. 2, *C* and *D*).

The Effect of Deletions within the Actin-binding Domain of α-Actinin on the Intracellular Localization of the Expressed Protein

The fact that targeting of α-actinin to actin-containing struc-

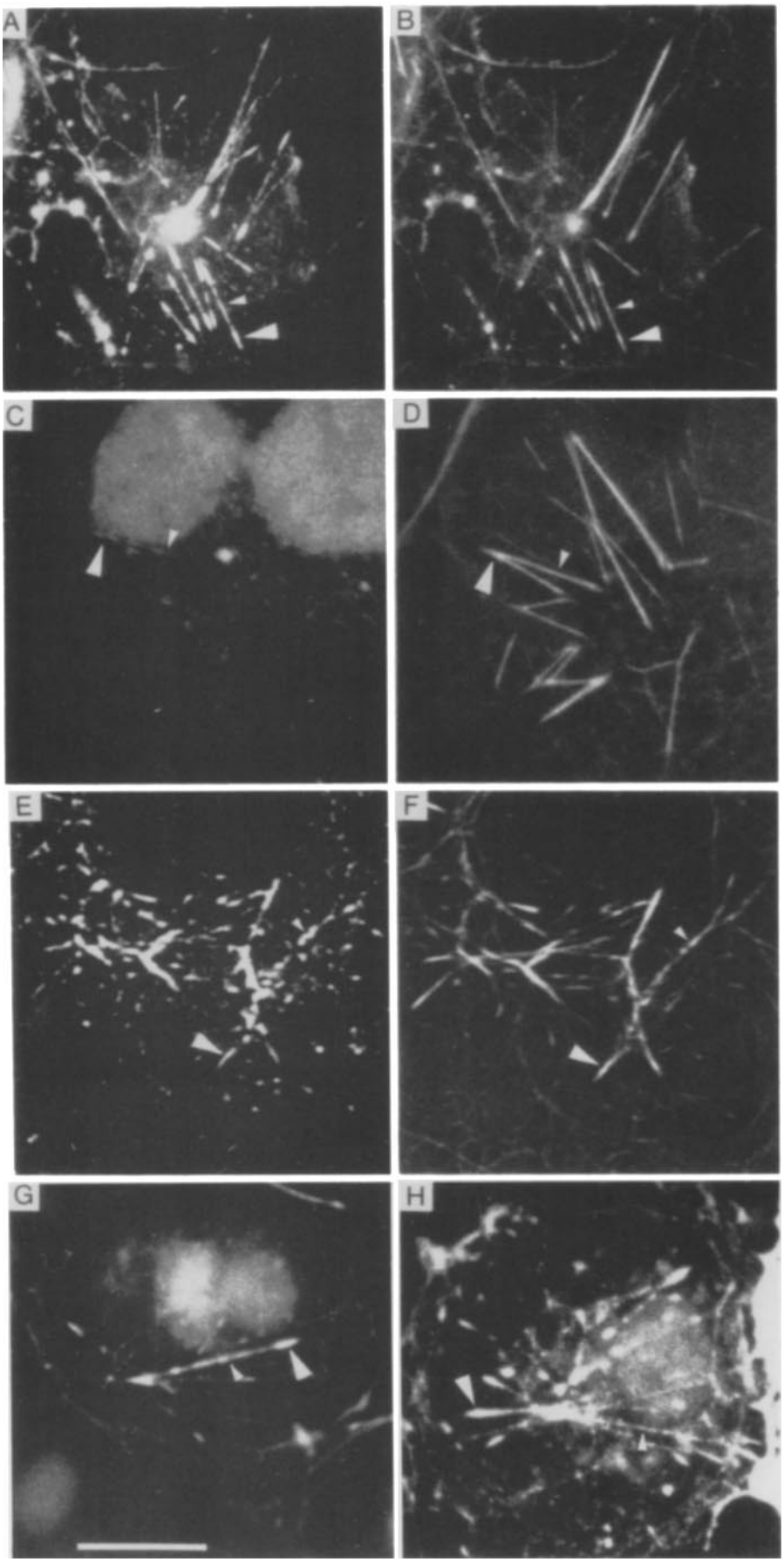

Figure 2. Expression of α-actinin and dystrophin cDNA constructs in Cos cells. Plasmid constructs expressed were pSVL/C17 (*A,B*), pSVL/N222 (*C,D*), and pSVL/DαA (*E–H*). Transfected cells were stained for the expressed chick α-actinins as described in Materials and Methods using a rabbit antibody to chick α-actinin (*A,C,E, G,H*). Double-staining for actin was achieved using NBD-phallacidin (*B,D,F*). Large arrowheads indicate the location of adhesion plaques and small arrowheads the location of actin filaments. Magnification bar, 5 μm.

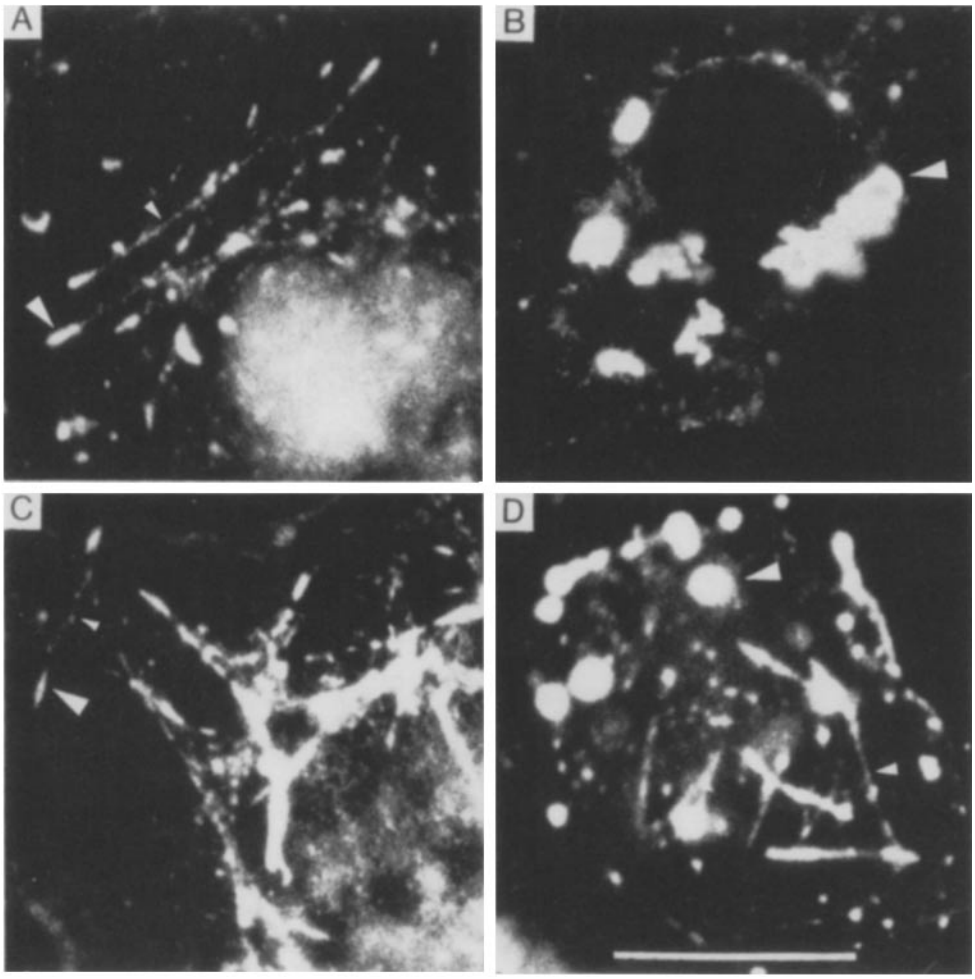

Figure 3. Expression of α-actinin mutants containing deletions within the actin-binding domain in Cos cells. The pSVL40 constructs expressed were: N20 (*A*), N47 (*B*), C216 (*C*), and C195 (*D*). Transfected cells were stained for α-actinin as described in Materials and Methods using a rabbit antibody to chick α-actinin. Large arrowheads indicate the location of adhesion plaques (*A*, *C*) and insoluble aggregates (*B*, *D*). Small arrowheads indicate the location of actin filaments. Magnification bar, 5 μm.

tures within the cell is dependent on the NH_2-terminal domain of the protein provides a system with which to further define the actin-binding site within this domain. We therefore constructed a number of α-actinin mutants in which nucleotide sequences encoding both NH_2- and COOH-terminal portions of the actin-binding domain were deleted. These mutants were subcloned into pSVL40 and transiently expressed in Cos cells. Deletion of NH_2-terminal residues 2–19 (pSVL/N20) did not affect the ability of the expressed α-actinin to localize to actin filaments or adhesion plaques (Fig. 3 *A*). In contrast, deletion of NH_2-terminal residues 2–46 (pSVL/N47) resulted in expression of a protein that was found in aggregates within the cytoplasm (Fig. 3 *B*). The result suggests that this deletion has led to synthesis of an insoluble protein, making it impossible to draw any conclusions from this particular mutant.

The expression and intracellular distribution of a deletion mutant lacking residues 217–242 at the COOH-terminal end of the actin-binding domain of α-actinin (pSVL/C216) is shown in Fig. 3 *C*. The expressed protein retained the ability to localize to adhesion plaques and actin stress fibers. An α-actinin molecule lacking residues 196–242 (pSVL/C195) was also able to localize to actin-containing structures (Fig. 3 *D*), but this deletion produced a marked alteration in the properties of the expressed protein, much of which was found in aggregates deposited throughout the cytoplasm. These results therefore suggest that the actin-binding site in α-actinin is contained within residues 20–195.

Expression of Truncated α-Actinins Containing the Actin-binding Domain in Cos Cells

In an attempt to overcome the solubility problems associated with expression of full-length α-actinins containing deletions within the actin-binding domain, we expressed truncated derivatives of these mutants spanning residues 1–290. Interestingly, the expressed wild-type protein encoded by the plasmid construct pSVL/ABD appeared to localize largely to the ends of actin filaments, and little was found distributed along the actin filaments themselves (Fig. 4, *A* and *B*). A mutant lacking residues 196–242 (pSVL/ABD195) also retained the ability to localize to filament ends (Fig. 4, *C* and *D*), consistent with our previous conclusion that these residues are not required for actin binding. However, much of the expressed protein remained diffusely distributed in the cytoplasm even though the cells had been permeabilized with detergent. α-Actinins that remain in the soluble pool are normally extracted under these conditions (Fig. 2 *C*), suggesting that the truncated mutant α-actinin (pSVL/ABD195) is only marginally more soluble than the full-length mutant (pSVL/C195).

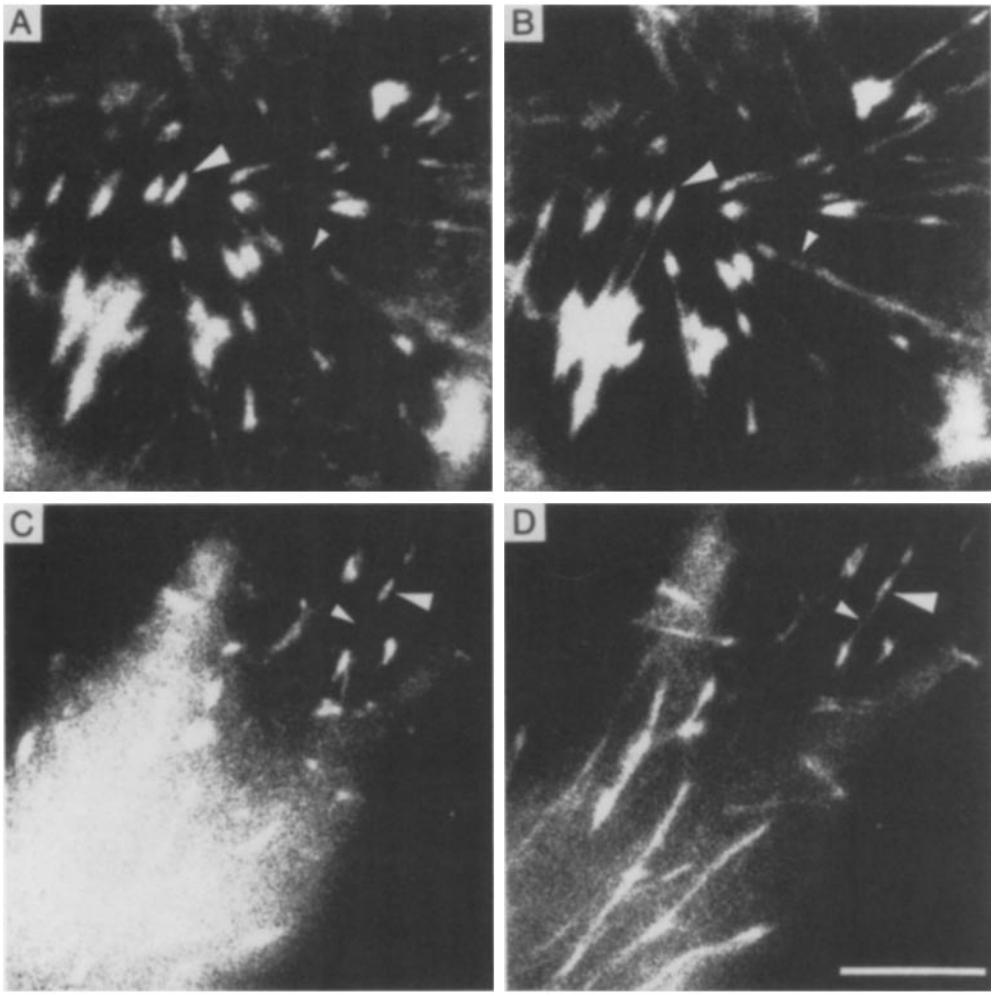

Figure 4. Expression of truncated α-actinins containing the actin-binding domain in Cos cells. The pSVL40 constructs expressed were: pSVL/ABD (*A*, *B*) and pSVL/ABD195 (*C*, *D*). Transfected cells were stained for α-actinin as described in Materials and Methods using a rabbit antibody to chick α-actinin (*A*, *C*). Double-staining for actin was achieved using NBD-phallacidin (*B*, *D*). Large arrowheads indicate the location of adhesion plaques and small arrowheads indicate the location of actin stress fibers. Magnification bar, 3 μm.

Expression of the Actin-binding Domain of α-Actinin as a Fusion Protein in E. coli and Analysis of Actin Binding In Vitro

The insolubility of α-actinin deletion mutants expressed in Cos cells precluded the use of this system to further define the actin-binding site in α-actinin. We therefore sought to express the actin-binding domain of α-actinin in *E. coli*. The expressed protein (residues 1–269) was soluble and was able to bind F-actin (data not shown). Unfortunately, all deletion mutants tested were insoluble, and we were unable to solubilize them from inclusion bodies using standard procedures. However, when deletion mutants were expressed as fusion proteins with GST using the pGEX expression system (Smith and Johnson, 1988), the proteins remained sufficiently soluble for purification and use in actin-binding studies. The ability of a fusion protein containing α-actinin residues 1–269 to cosediment with F-actin as revealed by SDS-PAGE and Coomassie blue staining of the gel is shown in Fig. 5 *b*. The fusion protein was found in the pellet (*p*) after centrifugation in the presence (+) of F-actin, but remained in the supernatant (*s*) when centrifuged in the absence (−) of actin. GST alone did not bind to F-actin (Fig. 5 *a*). The mutant fusion proteins tested were pGEX/N108, which contains α-actinin residues 108–242, and pGEX/C189, which contains α-actinin residues 1–189. Unfortunately, both fusion proteins have molecular weights similar to that of actin, and the proteins were therefore detected by Western blotting instead of staining with Coomassie blue. Interestingly, both pGEX/N108 (Fig. 6 *a*) and pGEX/C189 (Fig. 6 *b*) retained the ability to bind F-actin. A fusion protein containing residues 1–107 did not bind F-actin (data not shown). This result suggests that α-actinin residues 108–189 contain an actin-binding site.

Expression of α-Actinin KTFT Mutants in Cos Cells

The amino acid sequence KTFT in dystrophin (residues 19–22) has been implicated in binding to F-actin (Levine et al., 1990). This sequence is totally conserved among all α-actinins sequenced to date (residues 36–39 in chick smooth muscle α-actinin). To analyze the importance of this sequence in the binding of α-actinin to actin, we have expressed a number of α-actinin mutants affecting this region of the protein in Cos cells. Initially, we expressed an α-actinin construct in which nucleotides encoding these residues had been deleted. However, the expressed protein accumulated in aggregates, suggesting that it was insoluble (Fig. 7 *A*). Interestingly, cells expressing this construct were devoid of actin filaments (Fig. 7 *B*). We therefore went on to express mutants containing both conservative and nonconservative substitutions of the KTFT sequence. Despite the fact that this sequence is totally conserved in all α-actinins, substitution of the sequence with amino acids of similar physico-chemical prop-

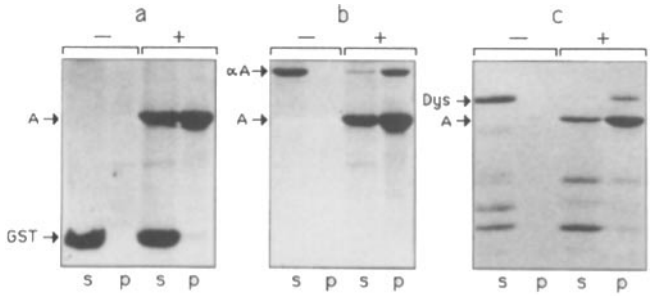

Figure 5. Binding of fusion proteins containing the NH_2-terminal domains of α-actinin and dystrophin to F-actin. GST or GST fusion proteins containing residues 1–269 of α-actinin (pGEX/αA) or residues 1–233 of dystrophin (pGEX/Dys) were expressed in and purified from *E. coli*, and their ability to bind F-actin was assayed using a cosedimentation assay. Proteins were incubated with (+) or without (−) actin before centrifugation, and the supernatants (*s*) and pellets (*p*) were analyzed by SDS-PAGE followed by staining with Coomassie blue. GST alone (*a*); GST fusion proteins containing the NH_2-terminal domains of α-actinin (*αA*) (*b*), and dystrophin (*Dys*) (*c*). The position of actin (*A*) and the fusion proteins is indicated (*arrows*).

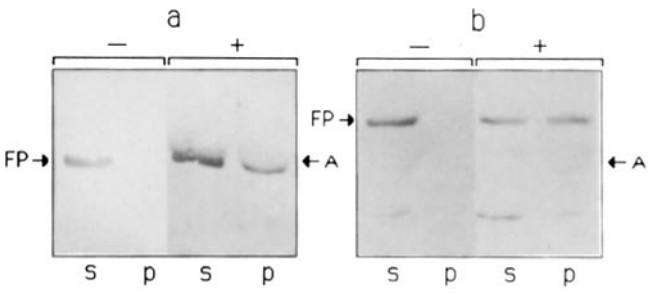

Figure 6. Binding of fusion proteins containing actin-binding domain deletion mutants to F-actin. Fusion proteins encoded by pGEX/N108 (residues 108–242; *a*) and pGEX/C189 (residues 1–189; *b*) were incubated with (+) or without (−) actin before centrifugation. The supernatants (*s*) and pellets (*p*) were analyzed by SDS-PAGE before detection of the fusion proteins by immunoblotting as described in Materials and Methods. The positions of the fusion proteins (*FP*) and actin (*A*) are indicated by arrows.

erties (RSYS) had no effect on the ability of the expressed α-actinin to localize to actin filaments and adhesion plaques (Fig. 7, *C* and *D*). Perhaps more surprising was the observation that the lysine residue plus a flanking arginine residue could be replaced by glutamate residues (EETFT) without any apparent effect on targeting of the expressed protein (Fig. 7 *E*). Three further mutations were made where residues 37, 38, and 39 (TFT) were each in turn substituted with alanine residues. Again, all three α-actinin mutants retained the ability to target to actin filaments and adhesion plaques (Fig. 7, *F–H*).

The Effect of Mutations in the KTFT Sequence on Actin Binding In Vitro

The actin-binding domains of the above KTFT mutants (residues 2–242) were also expressed as GST fusion proteins in *E. coli*, and their ability to bind to F-actin was analyzed in vitro. Sufficient soluble protein was obtained with all mutants, including the KTFT deletion mutant, although the majority of the expressed proteins proved to be insoluble (data not shown). All mutant fusion proteins were less stable than the wild-type protein, and the purified proteins had to be used immediately after purification. Nevertheless, the results clearly demonstrate that all intact fusion proteins (but not their degradation products) were capable of binding to F-actin, including the KTFT deletion mutant (Fig. 8). These results are consistent with those obtained from transfection experiments, and suggest that the KTFT motif is not essential for actin binding. Nevertheless, the amount of fusion protein bound to F-actin varied with the different mutations. In an attempt to quantify the relative binding activities of the different mutants, the amounts of each fusion protein bound to actin was measured by densitometry (Table I). Approximately 92% of the wild-type fusion protein sedimented in the presence (but not in the absence) of F-actin (Fig. 5 *b* and Table I). All mutants showed a substantial reduction in binding activity compared with the wild-type protein, although the differences in activities between the various mutants were surprisingly small.

Expression of a Dystrophin/α-Actinin Chimeric Protein in Cos Cells

The observation that the NH_2-terminal region of dystrophin shows sequence similarity to the actin-binding domain of α-actinin (Hammonds, 1987; Davison and Critchley, 1988) has led to speculation that dystrophin is also an actin-binding protein. To test this prediction, we constructed a chimeric cDNA encoding residues 1–233 of human dystrophin fused in frame to chick α-actinin residues 244–888. To facilitate the construction, the codon encoding serine residue 245 in the α-actinin sequence was mutated to an arginine codon. This substitution had no effect on the intracellular localization of the expressed α-actinin molecule (data not shown). When the construct encoding the dystrophin/α-actinin chimera (pSVL/DαA) was expressed in Cos cells, the fusion protein could clearly be seen to target to actin filaments and adhesion plaques (Fig. 2, *E–H*). This result shows that the NH_2-terminal domain of dystrophin is functionally homologous to that of α-actinin, and suggests that dystrophin is indeed an actin-binding protein.

Expression of the NH_2-terminal Domain of Dystrophin as a Fusion Protein in E. coli: Analysis of Its Actin-binding Properties In Vitro

When dystrophin residues 1–233 were expressed in *E. coli*, the protein was found in inclusion bodies from which it could not be solubilized. However, when these same residues were expressed as a GST fusion protein, sufficient protein remained in a soluble form to allow purification of the fusion protein. However, the fusion protein was somewhat unstable and had to be used in actin cosedimentation assays immediately after purification. The results of such experiments are shown in Fig. 5 *c*. The dystrophin fusion protein was almost exclusively recovered in the pellet fraction (*p*) when centrifuged in the presence (+) of F-actin, although it remained in the supernatant (*s*) fraction when centrifuged in the absence (−) of actin. It is therefore clear that the NH_2-terminal domain of dystrophin contains a functional actin-binding site.

Discussion

α-Actinin is one of a growing family of actin-binding pro-

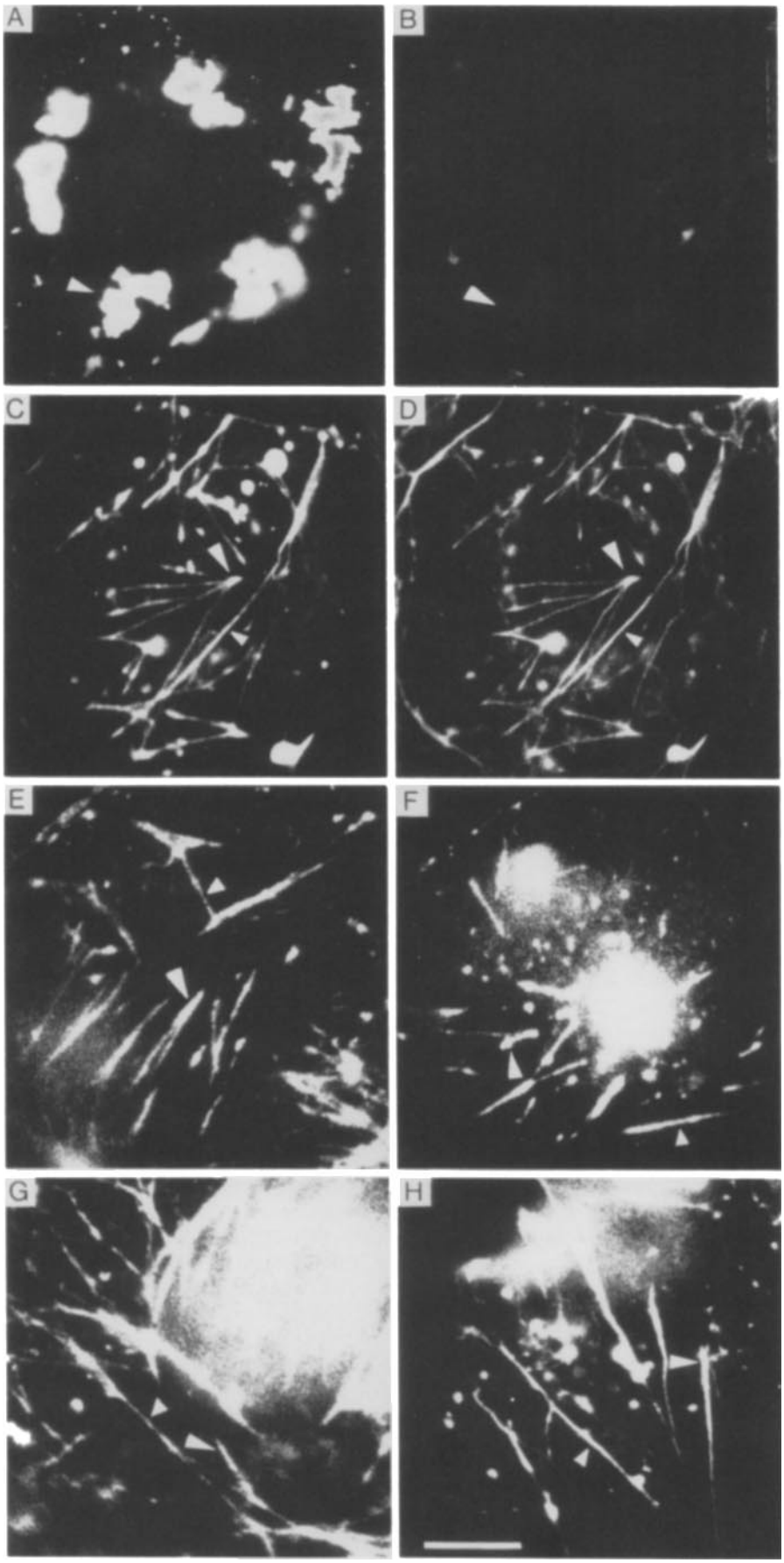

Figure 7. Expression of chick α-actinin KTFT mutants in Cos cells. pSVL/α-actinin cDNA constructs containing mutations in the R/K KTFT sequence (residues 35–39) were transiently expressed in Cos cells, and the expressed proteins were detected by immunofluorescence using a rabbit antibody to chick α-actinin (*A*, *C*, *E–H*). Double-staining for actin was achieved using NBD-phallacidin (*B*, *D*). Deletion of the KTFT sequence (*A*, *B*). Substitutions were as follows: **RSYS** (*C*, *D*), **EE**TFT (*E*), KA**F**T (*F*), KTA**T** (*G*), and KTF**A** (*H*). Large arrowheads indicate the location of adhesion plaques (*C–H*) and insoluble aggregates (*A*, *B*). Small arrowheads indicate the location of actin filaments (*C–H*). Magnification bar, 5 μm.

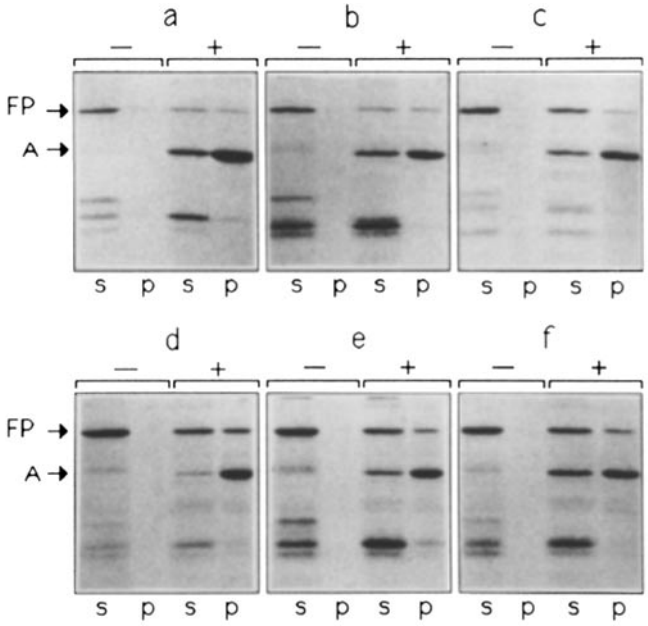

Figure 8. Binding of fusion proteins containing the actin-binding domain of α-actinin to F-actin. The effect of mutations in the KTFT sequence. Fusion proteins (α-actinin residues 2–242) were incubated with (+) or without (−) actin before centrifugation, and the supernatants (*s*) and pellets (*p*) were analyzed by SDS-PAGE and Coomassie blue staining. The positions of the fusion proteins (*FP*) and actin (*A*) are indicated by arrows. KTFT deletion mutant (*a*). Substitution mutants were as follows: RSYS (*b*), **EE**TFT (*c*), KAFT (*d*), KTAT (*e*), and KTFA (*f*).

teins that have homologous NH_2-terminal actin-binding domains (Matsudaira, 1991). Inspection of the aligned actin-binding domain sequences provides little information about the likely location of the actin-binding site within the domain (Fig. 9). There are 11 positions where sequence identity is maintained, 8 of which are hydrophobic amino acids. There are many other positions where the physico-chemical property of the residue is conserved. In an attempt to further define the actin-binding site in α-actinin, we have made a series of NH_2- and COOH-terminal deletions within the actin-binding domain, and have analyzed the effects of these deletions on actin binding in vivo and in vitro. Deletion of NH_2-terminal residues 2–19 of α-actinin had no apparent effect on the ability of α-actinin to target to actin filaments and filament ends when expressed in Cos cells. Deletion of

Table I. The Binding of α-Actinin Actin-binding Domain KTFT Mutant GST Fusion Proteins to F-Actin

GST fusion protein	Protein present in actin pellet
	%
Wild-type α-actinin actin-binding domain	92
KTFT deletion mutant	35
RSYS substitution mutant	52
EETFT substitution mutant	20
KAFT substitution mutant	48
KTAT substitution mutant	31
KTFA substitution mutant	25

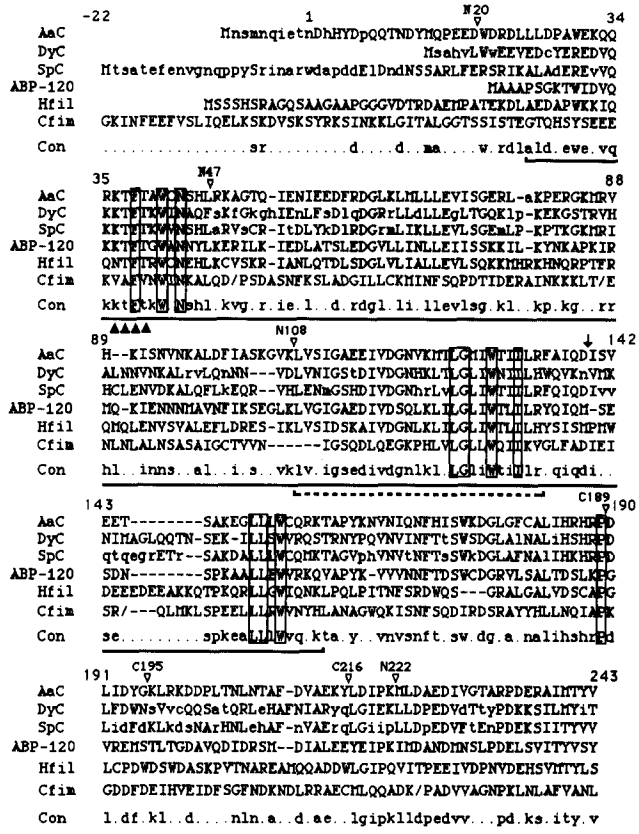

Figure 9. Amino acid sequence alignments of the actin-binding domains of the α-actinin family proteins. α-Actinin (*AaC*), dystrophin (*DyC*), β-spectrin (*SpC*), *Dictyostelium* ABP-120 (*ABP-120*) (Noegel et al., 1989), human filamin (*Hfil*) (Gorlin et al., 1990), and chick fimbrin (*Cfim*) (de Arruda et al., 1990). Optimal alignment was achieved using the computer program LINEUP (Devereux et al., 1984). The α-actinin consensus sequence (*AaC*) was derived from human placenta (Millake et al., 1989), chick smooth muscle (Baron et al., 1987), chick skeletal muscle (Arimura et al., 1988), *Drosophila* (Fyrberg et al., 1990), and *Dictyostelium* (Noegel et al., 1987) sequences. The dystrophin consensus sequence (*DyC*) was derived from human (Koenig et al., 1988) and chicken (Lemaire et al., 1988) sequences. The β-spectrin consensus sequence (*SpC*) was derived from human (Karinch et al., 1990) and *Drosophila* (Byers et al., 1989) sequences. Lowercase letters in the α-actinin, dystrophin, and β-spectrin consensus sequences represent positions where a consensus could not be derived, and the residues found in the chicken smooth muscle α-actinin, human dystrophin, and human β-spectrin sequences are shown at these positions. The consensus derived from all of these sequences (*Con*) is shown, where lowercase letters represent conserved residues, and uppercase letters represent identical residues (*boxed*). Dots represent positions where a consensus could not be derived. Residues −8 to −1 in the AaC are unique to chicken skeletal muscle α-actinin. The chicken fimbrin sequence starts at residue 69. Obliques inserted into the fimbrin sequence represent points where a number of nonhomologous residues have been omitted. The human β-spectrin tryptic peptide capable of actin-binding (Karinch et al., 1990) is indicated by a solid line. The 27 residues shown to be required for actin-binding in ABP-120 (Bresnick et al., 1990) are indicated by a dotted line. Solid arrowheads indicate the "KTFT" residues which have been implicated in the dystrophin/actin interaction (Levine et al., 1990). The extent of the deletion mutations at the NH_2- and COOH-terminal ends of the chicken smooth muscle α-actinin actin-binding domain are shown. The thermolysin protease cleavage site within chick skeletal muscle α-actinin is arrowed (↓). Numbering of residues is based on the chick smooth muscle α-actinin sequence including the initiating methionine.

NH_2-terminal residues 2–46, however, resulted in the expression of an insoluble protein. These residues are relatively hydrophilic, the remainder of the actin-binding domain being predominantly hydrophobic. Loss of these hydrophilic residues may permit intermolecular hydrophobic interactions which result in reduced solubility. Alternatively, deletion of these residues might produce a marked change in conformation and therefore solubility of the protein, although a protein lacking almost the entire actin-binding domain (pSVL/N222) was soluble. Deletion of residues 217–242 and 196–242 at the COOH terminus of the actin-binding domain was more informative, and both mutants retained the ability to localize to actin filaments and filament ends. From these results we can conclude that the actin-binding site in α-actinin lies between residues 20 and 195. The conclusion that the extreme NH_2-terminal region of α-actinin is not required for binding is consistent with the observation that the 27-kD polypeptide released from chick α-actinin by thermolysin is able to bind to actin (Mimura and Asano, 1986) although it lacks residues 1–24 (Davison et al., 1989). Furthermore, the extreme NH_2-terminal region of this family of actin-binding proteins shows no sequence relatedness (Fig. 9), making it unlikely to be involved in binding to the highly conserved actin molecule.

Our initial attempts to further define the actin-binding site in α-actinin using bacterially expressed proteins were encouraging. Thus a protein (residues 1–269) containing the intact actin-binding domain remained soluble, and was able to bind F-actin in vitro. The expressed actin-binding domain deletion mutants accumulated in inclusion bodies from which they could be solubilized using 6 M urea. However, we were unable to identify conditions to maintain them in solution after removal of the urea. We were able to overcome this problem to some degree by expressing deletion mutants as fusion proteins with GST. This approach has the added advantage that the proteins can be purified by affinity chromatography using glutathione-agarose. The results of these studies clearly demonstrate that NH_2-terminal residues 1–107 and COOH-terminal residues 190–242 are not required for actin binding. The actin-binding site in α-actinin must therefore lie between residues 108 and 189.

It is interesting to compare these results with those gained from studies on other members of this family of proteins. A 16.5-kD tryptic polypeptide derived from human β-spectrin has recently been demonstrated to bind F-actin (Karinch et al., 1990). The polypeptide spans residues 47–186 in the β-spectrin sequence which are equivalent to residues 25–158 in the chick smooth muscle α-actinin sequence (Fig. 9). In addition, a 17-kD tryptic fragment derived from ABP-120 has also been reported to bind F-actin, whereas a 14-kD fragment generated from this polypeptide was found to have lost actin-binding activity (Bresnick et al., 1990). Protein sequence analysis showed that the NH_2 termini of the 17- and 14-kD fragments were residues 89 and 115, respectively. The authors conclude that the sequence between residues 89 and 115 is essential for actin binding. These 27 residues in ABP-120 are equivalent to residues 108–134 in the chick smooth muscle α-actinin sequence (Fig. 9). They are therefore contained within the sequence in α-actinin (residues 108–189) we have shown to be important in binding to actin. The sequence is also contained within the region in β-spectrin thought to be important in actin binding (Karinch et al., 1990). Furthermore, these 27 residues are highly conserved in all members of this family of actin-binding proteins (Bresnick et al., 1990). The totally conserved residues within this sequence are predominantly hydrophobic in nature. This may indicate that binding of the α-actinin family of proteins to actin involves predominantly hydrophobic interactions, although it will be important to establish that these residues are displayed on the surface of the protein.

Unfortunately, there appears to be no experimental evidence concerning the nature of the interaction between actin and members of this family of actin-binding proteins. It is interesting to note that two possible α-actinin binding sites have been identified on the surface of actin using chemical crosslinking (Mimura and Asano, 1987). The first of these sites is between actin residues 1 and 12, which are mainly acidic in nature. The second site is between residues 86 and 123. Actin residues 90–103 have been shown to form a surface loop (Kabsch et al., 1990) that is moderately hydrophilic in nature (Collins and Elzinga, 1975). Whether the 27 predominantly hydrophobic residues implicated in actin binding from studies on ABP-120 (Bresnick et al., 1990) interact with these regions of the actin molecule remains to be established.

Evidence has been presented that the NH_2-terminal region of this family of actin-binding proteins arose by a gene duplication event (de Arruda et al., 1990). Thus residues 18–140 of chick smooth muscle α-actinin can be aligned with residues 141–245. Interestingly, the actin-binding domain of chick skeletal muscle α-actinin can be cleaved almost exactly in half by thermolysin (between aspartate 139 and isoleucine 140 using the coordinates of the smooth muscle isoform; see Fig. 9) (Davison, M. D., and D. R. Critchley, unpublished data), supporting the view that the domain is composed of two distinct segments. Whether the actin-binding site is contained solely within the NH_2-terminal segment, as one might conclude from the studies on ABP-120, or extends into the COOH-terminal segment remains to be established.

Nuclear magnetic resonance experiments with a synthetic peptide spanning residues 10–32 of the human dystrophin sequence suggest that it contains an actin-binding site (Levine et al., 1990). Further, the sequence KTFT which is contained within this peptide has been shown to interact with actin. The sequence R/K KTFT is found in human, chick, and *Dictyostelium discoideum* α-actinins, human and chick dystrophin, human and *Drosophila* β-spectrin, and *Dictyostelium* ABP-120, but the sequence is somewhat divergent in fimbrin and filamin (Fig. 9). We have attempted to clarify the importance of this sequence in the interaction between α-actinin and actin by mutagenesis. Deletion of this sequence (residues 36–39 in the chick smooth muscle protein) resulted in the expression of an apparently insoluble protein in Cos cells, but conservative and nonconservative substitutions did not affect the ability of expressed α-actinin to target to actin filaments or filament ends. In addition, when the actin-binding domains of the KTFT mutants, including the deletion mutant, were expressed as fusion proteins in *E. coli*, the proteins were still able to bind F-actin. We therefore conclude that this sequence is not essential to actin binding, although we do not exclude the possibility that it contributes to the interaction. Indeed, there was an apparent reduction in the ability of the KTFT mutants to cosediment with actin, although we cannot rule

out the possibility that this was due to the instability of these proteins. In common with a number of other actin-binding proteins, α-actinin has been reported to bind to the acidic NH_2-terminal region of actin (Mimura and Asano, 1987). The basic residues in the sequence R/K KTFT may be important in this respect.

Although the NH_2-terminal region of human dystrophin shows a 45% sequence identity to the actin-binding domain of human α-actinin (67% sequence similarity) (Fig. 9), there is no direct evidence that dystrophin does in fact bind actin. The experiments described in this study show for the first time that the NH_2-terminal region of dystrophin is indeed functionally homologous to that of α-actinin. Thus, a chimeric protein in which the actin-binding domain of chick α-actinin was replaced by residues 1–233 of human dystrophin localized to actin filaments and filament ends when expressed in Cos cells. A bacterially expressed fusion protein containing dystrophin residues 1–233 also bound F-actin in vitro. These results provide unequivocal evidence that dystrophin is an F-actin binding protein.

The ability of the NH_2-terminal region of dystrophin to bind to actin is apparently at variance with the observed intracellular distribution of the protein. Thus in skeletal muscle, dystrophin is localized under the plasma membrane and is not, for example, directly associated with the actin thin filaments of the contractile apparatus (Bonilla et al., 1988; Watkins et al., 1988). Furthermore, expression of the whole dystrophin molecule in Cos cells leads to targeting of the expressed protein to the cell periphery, and not to the actin stress fibers within the cell (Lee et al., 1991). It would therefore seem likely that the dystrophin molecule contains additional sequences that are important in determining the intracellular distribution of the protein. Dystrophin has been shown to bind to a membrane glycoprotein found in the skeletal muscle membrane (Ervasti et al., 1990), and this might be an important factor governing its location within the cell. However, the region of the dystrophin molecule involved in this interaction has yet to be defined. Interestingly, α-actinin has also been reported to contain a binding site for an integral membrane protein, and this site has been localized to the four spectrin-like repeats contained within the molecule (Otey et al., 1990). These repeats are homologous to the 24 such repeats found in dystrophin (Davison and Critchley, 1988; Koenig et al., 1988).

The authors are grateful to Professor R. C. Griggs (University of Rochester, Rochester, NY) for his help in making the dystrophin actin-binding domain construct in pGEX-2, and to Dr. K. Davies (University of Oxford, Oxford, UK) for making the dystrophin cDNA available. We are also grateful to Dr. A. Weeds, Dr. M. Wey, and Mr. B. Pope (Medical Research Council Molecular Biology Laboratories, Cambridge, UK) for their generous help and advice throughout the project.

The work was funded by the Medical Research Council (UK).

Received for publication 20 June 1991 and in revised form 4 December 1991.

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