

The identification and characterisation of an actin-binding site in α -actinin by mutagenesis

Philip A. Kuhlman, Lance Hemmings and David R. Critchley

Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 7RH, UK

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We have shown previously that the N-terminal actin-binding domain of α -actinin retains activity when expressed in *E. coli* as a fusion protein with glutathione-S-transferase. In the present study we have made a series of N- and C-terminal deletions within this domain and show that an actin-binding site is contained within residues 120–134. Amino acid substitutions within this region indicate that several highly conserved hydrophobic residues are involved in binding to F-actin. The hypothesis that the interaction between α -actinin and F-actin is predominantly hydrophobic in nature is supported by the observation that binding is relatively independent of salt concentration.

α -Actinin; Actin-binding site; Dystrophin; β -Spectrin; ABP-120; Cytoskeleton

1. INTRODUCTION

α -Actinin is a member of a family of F-actin-binding proteins which share a homologous N-terminal actin-binding domain [1]. Other members of the family include β -spectrin [2], dystrophin [3], filamin [4], and the *Dictyostelium discoideum* actin gelation factor ABP-120 [5]. The proteins fimbrin [6] and adducin [7] also contain sequences which show some homology to this region of α -actinin. In a recent study, we expressed α -actinin residues 1–269 in *E. coli* as a fusion protein with glutathione-S-transferase (GST) and showed that the protein retained the ability to bind to F-actin [8]. Furthermore we demonstrated that there is an actin binding site located between α -actinin residues 108–189. In an attempt to further define those residues which are important in binding actin, we have expressed a number of additional fusion proteins containing various regions of the N-terminal domain of the α -actinin molecule. The results of this study show that an actin-binding site in α -actinin is located between residues 120–134 of the chick smooth muscle isoform [9].

2. MATERIALS AND METHODS

2.1. Expression of the actin-binding domains of mutant α -actinins as fusion proteins with glutathione-S-transferase (GST)

The deletion mutant constructs shown in Fig. 1 (with the exception

of pGEX/C189) were generated by PCR using the smooth muscle α -actinin cDNA clone C17 [9] as template and the appropriate primers. The 5' oligonucleotide primers contained *Bam*HI restriction enzyme cleavage sites and the 3' primers either *Eco*RI or *Bam*HI cleavage sites enabling the PCR products to be cloned into either *Bam*HI/*Eco*RI or *Bam*HI cut pGEX-2 (Pharmacia). The construct encoding α -actinin residues 1–189 fused to GST (pGEX/C189) was made by cutting the pGEX/C217 construct (Fig. 1) at a convenient *Sac*I site within the actin-binding domain, and at a 3' *Eco*RI site within the pGEX-2 polylinker. Following removal of the *Sac*I–*Eco*RI fragment, the ends were made flush using T4 DNA polymerase I, and the vector religated. Actin-binding domain constructs containing internal deletions or point mutations were generated by site-directed mutagenesis using the C17 cDNA cloned into the M13 derivative MICE mp18 [10] as template. The frequency of mutant isolation was increased by generating a single strand template using the *dut*[–] *ung*[–] *E. coli* strain [11]. Mutants were identified by T-track dideoxynucleotide sequencing. DNAs encoding residues 1–269 and containing the various mutations were generated from the mutant C17 cDNAs by PCR amplification using appropriate 5' and 3' primers. PCR products were subcloned into *Bam*HI–*Eco*RI cut pGEX-2. Recombinants were identified by the expression of fusion proteins of the correct size. 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 using glutathione-agarose essentially as described by Smith and Johnston [12] with slight modification. The salt concentration of the PBS buffer was raised to 200 mM and Triton X-100 (0.1% v/v) was added to the PBS to aid solubility of the proteins. The fusion proteins were eluted from a glutathione-agarose affinity matrix using 5 mM glutathione in PBS. The concentration of fusion proteins was established by densitometric scanning of SDS-PAGE gels of the fusion proteins along with known concentrations of G-actin.

2.2. Actin co-sedimentation assay

The ability of the α -actinin fusion proteins to bind F-actin was evaluated by a co-sedimentation assay exactly as described previously [8]. Supernatants and pellets were analysed by SDS-PAGE followed by Coomassie blue staining. In some cases the size of the fusion protein was such that it was difficult to resolve from actin. In these cases, proteins were transferred to nitrocellulose and the fusion proteins

Abbreviations: GST, glutathione-S-transferase; PBS, phosphate-buffered saline.

Correspondence address: D.R. Critchley, Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 7RH, UK.
Fax: (44) (533) 523369.

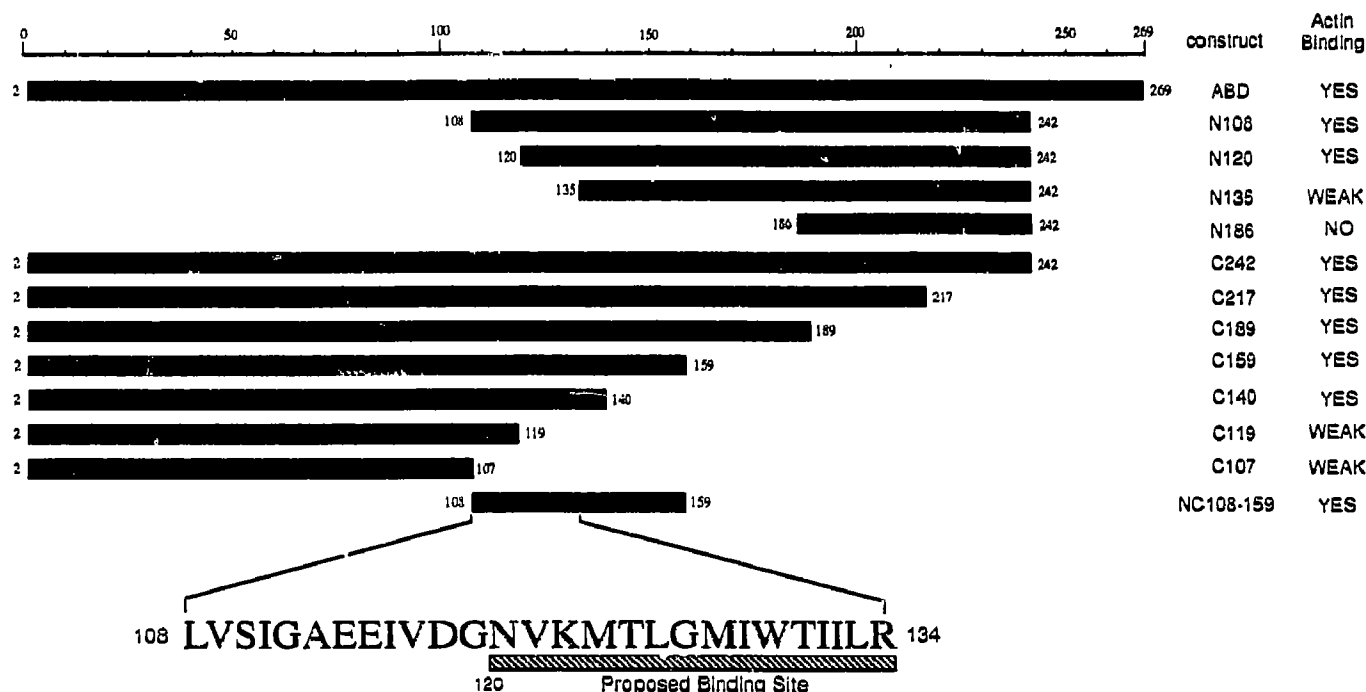


Fig. 1. α -Actinin actin-binding domain deletion mutants expressed as fusion proteins with glutathione-S-transferase (GST). The residue numbers of the N and C termini of the proteins are shown. Numbering includes the initiating methionine and is based on the sequence of chick smooth muscle α -actinin [9]. The names of the α -actinin constructs and their ability to bind F-actin are indicated together with the amino acid sequence of the actin-binding site identified in this study.

detected by Western blotting using a polyclonal antibody to chicken gizzard α -actinin, again as described previously [8].

3. RESULTS

3.1. Analysis of the ability of actin-binding domain deletion mutants to bind F-actin

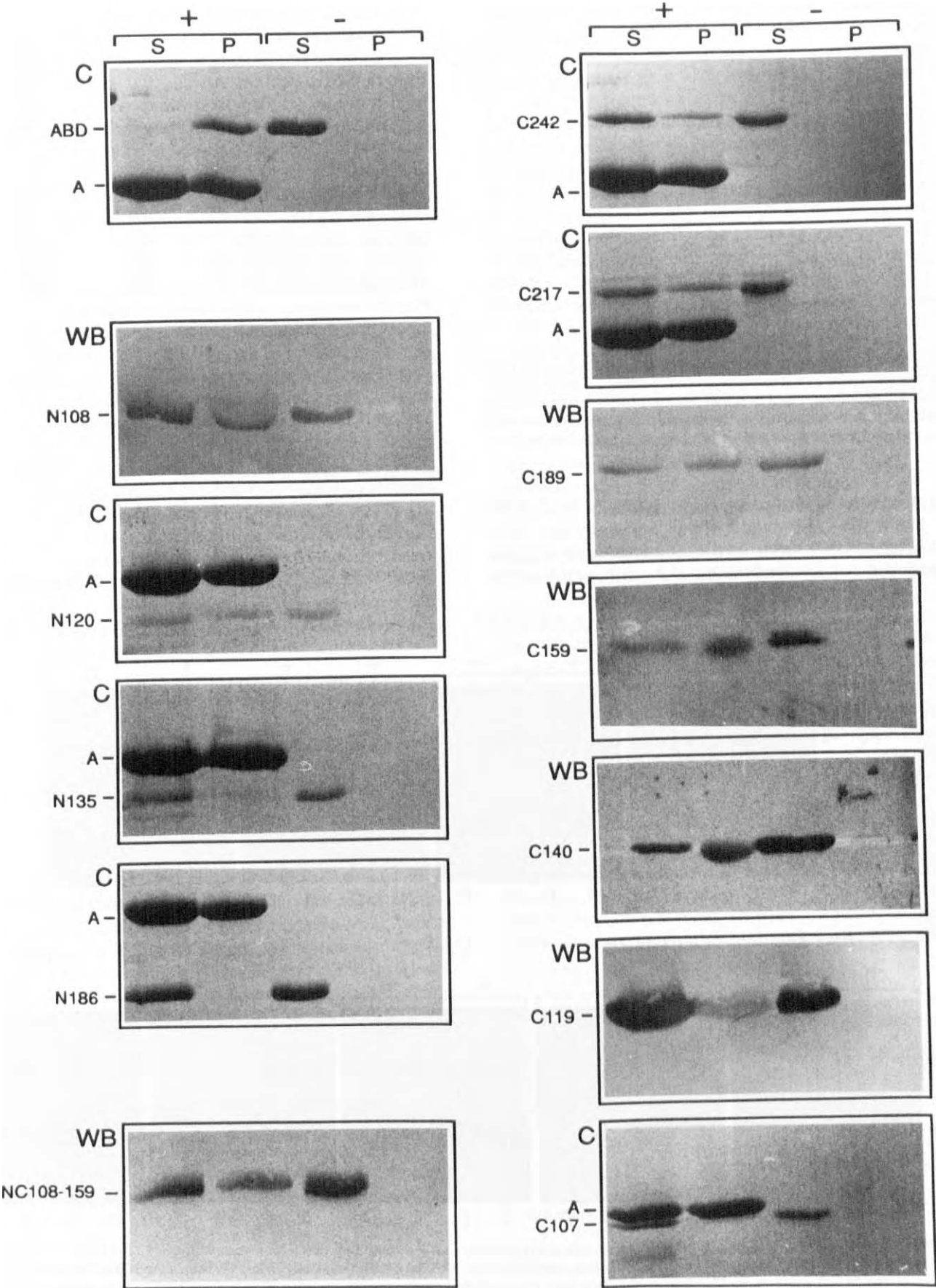
We have shown previously that a GST fusion protein containing chick smooth muscle α -actinin residues 1–269 can bind to F-actin as determined by a co-sedimentation assay (reference [8] and Fig. 2; ABD). GST alone does not bind F-actin [8]. In order to further define the actin-binding site within α -actinin, we have expressed a series of actin-binding domain deletion mutants as GST fusion proteins (Fig. 1) and tested their ability to bind F-actin (Fig. 2). Mutants lacking N-terminal residues 1–107 (N108) and 1–119 (N120) still retained the capacity to bind to F-actin, although more of the fusion protein remained in the supernatant than was the case with the wild type ABD (Fig. 2). However, a mutant lacking N-terminal residues 1–134 (N135) showed almost complete loss of binding activity, and mutant N186 was devoid of activity. Fusion proteins with progressive C-terminal deletions up to residue 140 retained the ability

to bind to F-actin (Fig. 2; C242, C217, C189, C159, C140). However, further C-terminal deletions (C119 and C107) resulted in a dramatic reduction in binding. These results suggest that there is an F-actin binding site between residues 120–134 in α -actinin. In an attempt to confirm this result, we expressed a GST fusion protein containing α -actinin residues 108–140, but unfortunately the protein was insoluble. However, a slightly larger fusion protein containing residues 108–159 was soluble, and was indeed capable of binding F-actin as predicted from the above results (Fig. 2; NC108-159).

3.2. The effect of increasing salt concentration upon binding of GST/ABD to F-actin

The amino acids found within residues 120–134 of α -actinin are predominantly hydrophobic suggesting that binding to actin might be mediated by hydrophobic interactions. To test this hypothesis, we investigated the effect of increasing salt concentration on the binding of GST/ABD to F-actin (Fig. 3). Increasing the salt concentration reduced the amount of GST/ABD and actin recovered in the pellet fraction. The ratio of GST/ABD (line gradient = -18.2) to actin (line gradient = -16.7) recovered in the pellet was very similar at all salt con-

Fig. 2. Binding of GST fusion proteins containing the various actin-binding domain deletion mutants to F-actin analysed by co-sedimentation. Fusion proteins were incubated with (+) or without (-) actin before centrifugation. The supernatants (S) and pellets (P) were analysed by SDS-PAGE before detection of the fusion proteins by Coomassie blue staining (C) or Western blotting (WB). The position of the fusion proteins and actin (A) are indicated.



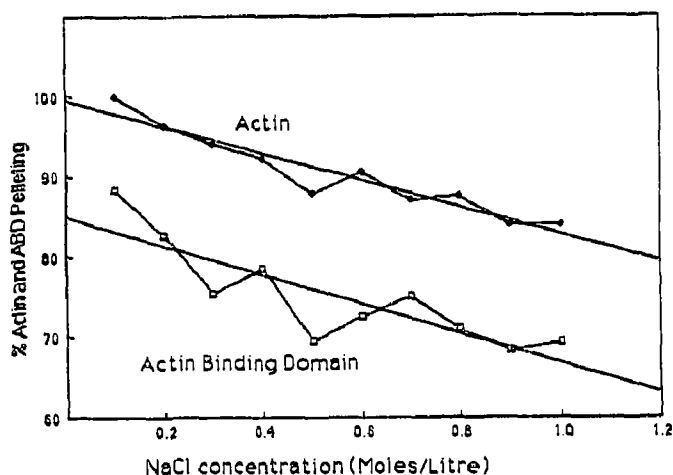


Fig. 3. The effect of increasing salt concentrations on binding of a GST fusion protein (ABD) containing α -actinin residues 1-269 to F-actin. The percentage of actin and α -actinin fusion protein co-sedimenting was estimated by densitometry of Coomassie blue stained SDS-PAGE gels on which supernatants and pellets had been analysed following incubation with or without actin.

centrations tested indicating that binding of GST/ABD to F-actin was independent of salt concentration. This result suggests that electrostatic interactions do not play a significant role in the binding of α -actinin to F-actin.

3.3. The effect of mutations in residues 108-134 on the binding of GST/ABD to F-actin

Alignment of the amino acid sequences of the α -actinin family of proteins reveals that residues 108-134 are highly conserved amongst all members of the family. Several residues within this sequence are totally conserved whilst the physico-chemical properties of others are maintained. In order to further characterise this actin-binding site in α -actinin, several mutations were made within this conserved sequence, and the effect of these mutations on the ability of a fusion protein containing residues 1-269 (GST/ABD) to bind to F-actin investigated using the co-sedimentation assay (Fig. 4). Equal amounts of each fusion protein were used in the assay thereby maintaining an approximate molar ratio of fusion protein to actin of 1:6. Densitometry was used to estimate the percentage of fusion protein bound to F-actin (Fig. 5). Deletion of residues 111-117 (Δ 111-117) outside the region implicated in binding produced a relatively small decrease in activity (28%) (Fig. 4; Δ 111-117: Fig. 5). Charged residue Asp-118 was substituted for alanine and although some reduction in binding (33%) did result from this change (Fig. 4; DA118: Fig. 5) the effect was not as great as that seen following substitution of hydrophobic residues Leu-125, Met-127, Ileu-128, Trp-129, Ileu-131 and Ileu-132 (Fig. 4; LA125,

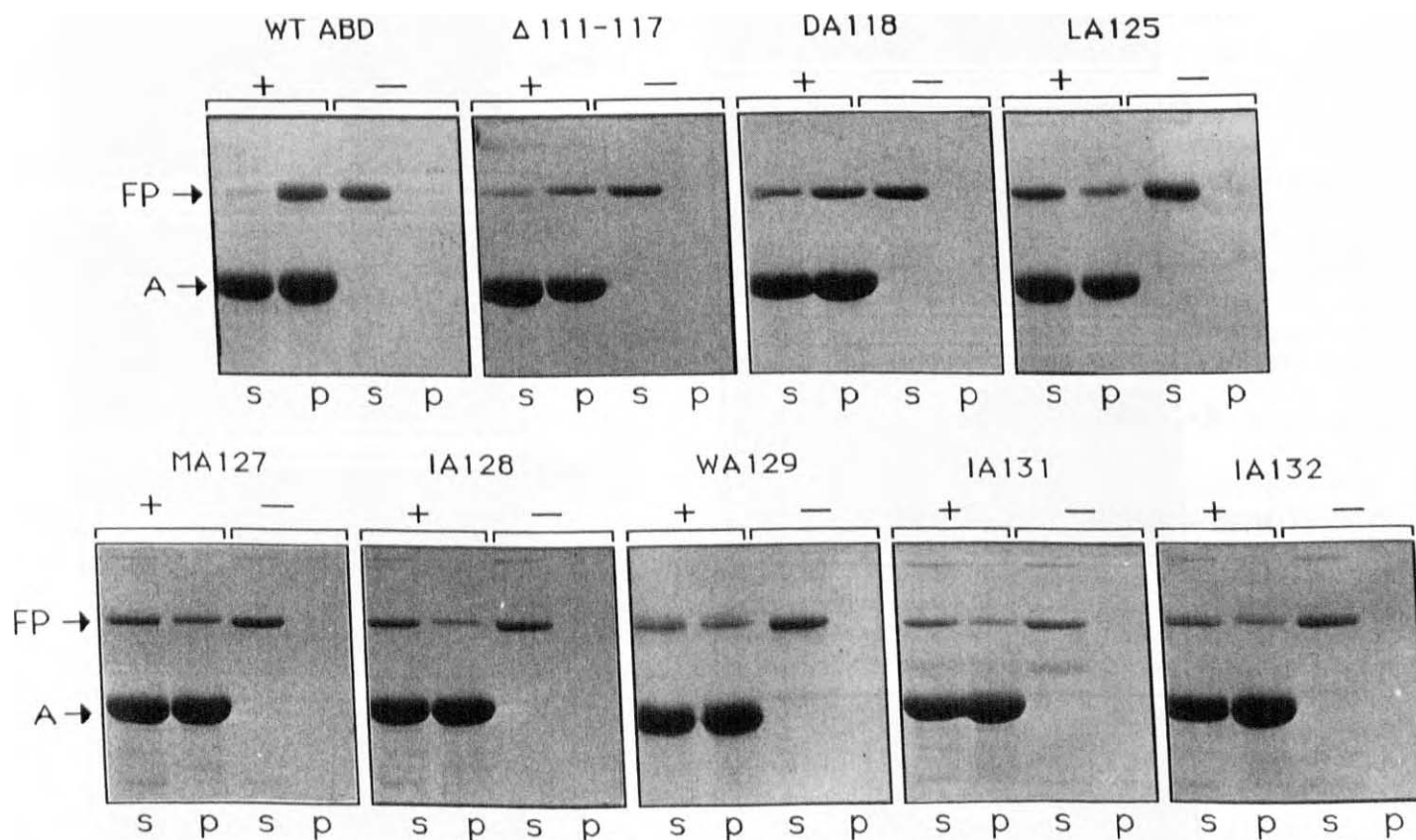


Fig. 4. Binding of GST/ABD fusion proteins containing internal deletions and alanine substitutions within residues 108-134 to F-actin. Fusion proteins were incubated with (+) or without (-) actin before centrifugation. The supernatants (s) and pellets (p) were analysed by SDS-PAGE before detection of the fusion proteins by Coomassie blue staining. The positions of the fusion proteins (FP) and actin (A) are indicated by arrows.

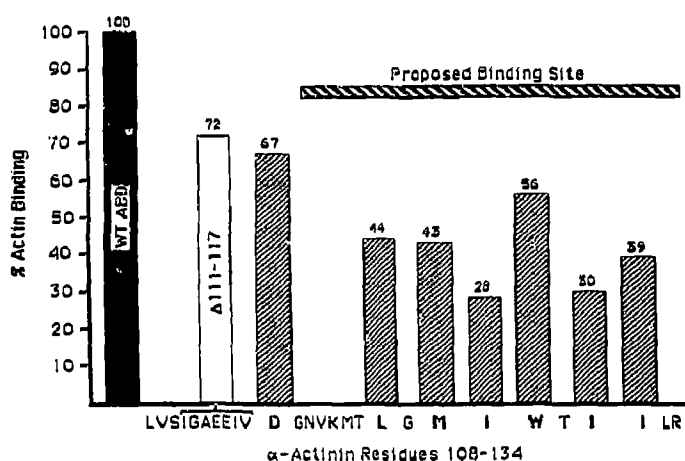


Fig. 5. Quantitative analysis of the binding of GST/ABD deletion and point mutants to F-actin. The percentage of the fusion protein bound to actin in Fig. 4 was estimated by densitometry of Coomassie blue stained SDS-PAGE gels on which supernatants and pellets had been analysed. WT ABD, α -actinin residues 1-269; Δ 111-117, an ABD deletion mutant lacking residues 111-117. α -Actinin residues 108-134 are shown at the bottom of the figure. Residues substituted with alanine are shown in bold type. The extent of the proposed binding site (residues 120-134) is indicated.

MA127, IA128, WA129, IA131 and IA132: Fig. 5). These results support the conclusion that the interaction between α -actinin and F-actin is predominantly hydrophobic in nature.

4. DISCUSSION

We have used a series of overlapping GST fusion proteins based on α -actinin residues 1-269 to identify an actin-binding site within the protein to between residues 120-134. Thus, fusion proteins containing α -actinin residues 2-140 and 120-242 retained the ability to co-sediment with F-actin whereas those containing residues 2-119 and 135-242 showed little if any activity. Confirmation that this region of α -actinin does indeed contain a functional actin binding site was obtained by the demonstration that a fusion protein containing residues 108-159 was able to bind F-actin. These results are entirely consistent with studies on two other members of the α -actinin family of actin-binding proteins. A 16.5-kDa tryptic fragment of human β -spectrin, equivalent to residues 25-158 in chick smooth muscle α -actinin, has been shown to bind F-actin [2]. Similarly, a 17-kDa tryptic fragment from ABP-120 retained actin-binding activity, but a 14-kDa fragment derived from it by loss of 27 amino-terminal residues was without activity [13]. Interestingly, these 27 residues in ABP-120 are equivalent to residues 108-134 in chick smooth muscle α -actinin, and overlap the actin-binding site in α -actinin identified in this study. A synthetic 27mer containing these residues inhibited actin cross-linking by ABP-120, and the peptide was able to co-sediment with

F-actin [14]. Furthermore, antibodies to the synthetic 27mer cross-reacted with native ABP-120 clearly establishing the surface orientation of at least a part of this region of the protein [14]. This region is highly conserved in all members of this family of actin-binding proteins, and is encoded by a single exon in dystrophin [3] suggesting that it might comprise a discrete domain. The conserved residues are predominantly hydrophobic in nature. Mutations in which a number of these conserved hydrophobic residues in α -actinin were replaced individually with alanine produced a substantial reduction in actin-binding consistent with the conclusion that they are directly involved in the interaction with actin. The fact that the interaction between α -actinin residues 1-269 and F-actin is insensitive to increasing salt concentration is also consistent with the view that binding is predominantly hydrophobic in nature. Hydrophobic interactions have also been implicated in the binding of myosin II to actin [15].

Cross-linking studies suggest that α -actinin interacts with actin residues 1-12 and 86-123 [16] as well as residues close to the C terminus of actin [17]. It is therefore likely that other sequences within the N-terminal region of α -actinin, apart from residues 120-134, contribute to the interaction with actin. Two putative actin-binding sites have been identified within the N-terminal domain of dystrophin based on NMR studies [18,19]. One site is equivalent to smooth muscle α -actinin residues 25-49, and contains the sequence KTFT which is conserved in most members of the α -actinin family of actin-binding proteins. However, α -actinin deletion mutants lacking this sequence are still able to bind actin [8]. The other site is equivalent to smooth muscle α -actinin residues 141-167. It is possible that these two sites account for the small amounts of the fusion proteins C107 and N135 which are found to co-sediment with actin, although the levels are such that we cannot exclude non-specific trapping of the proteins. It will be important to measure the binding affinities of the various α -actinin deletion mutants for F-actin. Such an approach may reveal sites outside of residues 120-134 which contribute to the interaction between the two proteins.

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