Calcium/calmodulin-dependent regulation of the NH_2-terminal F-actin binding domain of utrophin

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Abstract The cytoskeletal proteins utrophin, dystrophin and α-actinin are predicted to form antiparallel dimers thus potentially bringing their NH_2-terminal F-actin binding domains in close proximity to their EF-hand containing COOH-terminal domains. This arrangement would allow for calcium-dependent regulation of F-actin binding. We tested this hypothesis by determining the effect of the ubiquitous calcium binding protein calmodulin on their F-actin binding capabilities. Binding of the NH_2-terminal F-actin binding domain of utrophin to F-actin was inhibited by increasing concentrations of calmodulin in a calcium-dependent manner. The homologous F-actin binding domains from dystrophin and α-actinin were not regulated by calmodulin in the presence or absence of calcium. These findings have implications for the structural organisation of utrophin dimers and also for the replacement of dystrophin by over-expression of utrophin in dystrophic muscle.

Key words: Utrophin; Dystrophin; α-Actinin; Actin binding; Calcium–calmodulin; Regulation

1. Introduction

The structurally related cytoskeletal proteins αactinin, dystrophin, utrophin and spectrin, contain both F-actin binding and calcium binding domains. Dystrophin, utrophin and α-actinin have both domains in one molecule, whereas α-spectrin contains the EF-hand calcium binding regions, and β-spectrin contains the F-actin binding site. In the case of α-actinin homodimers and spectrin heterodimers, the EF-hand containing regions are in close proximity to the F-actin binding domains and are believed to exert a calcium-dependent regulatory effect on their F-actin binding [1,2]. Electron microscopic data indicates that dystrophin may exist as dimers [3,4], and based on the high degree of homology between dystrophin and utrophin, one could assume that utrophin also forms dimers. It is not clear, however, whether these dimers are parallel or antiparallel. If by analogy with α-actinin they are antiparallel, then it is possible that the NH_2-terminal F-actin binding domains of dystrophin and utrophin could be regulated in a calcium-dependent manner by the COOH-terminal EF-hand region of an adjoining monomer. The arrangement of EF-hand structures in this group of proteins resemble that of the ubiquitous calcium-binding protein calmodulin. Although examination of their sequences suggests that dystrophin and utrophin EF-hands may not bind calcium [5,6], an expressed COOH-terminal dystrophin construct did bind calcium in a gel overlay assay [7]. Furthermore, recent structural evidence has shown that the scallop myosin essential light chain contains a specific calcium binding site even though sequence data suggested strongly against this site binding calcium [8]. We have therefore tested the ability of the ubiquitous and structurally related calcium binding protein calmodulin to regulate the binding of the bacterially expressed F-actin binding domains of α-actinin, dystrophin and utrophin to F-actin.

2. Materials and methods

The constructs containing the NH_2-terminal F-actin binding domains of α-actinin, dystrophin and utrophin are designated AAC140 (residues 1–140 of α-actinin [9]), DMD246 (residues 1–246 of dystrophin [10]) and UTR261 (residues 1–261 of utrophin [11]). They were expressed in *E. coli* and purified from cell lysates as previously described [9–11]. Calmodulin was prepared from porcine brain [12] and actin from rabbit skeletal muscle [11].

F-Actin binding sedimentation assays were carried out as described previously [11]. Briefly; AAC140 (22 μM), DMD246 (18.4 μM) and UTR261 (19.6 μM) were incubated with increasing concentrations of calmodulin (5–150 μM) in the presence or absence (presence of 1 mM EGTA) of 1 mM $CaCl_2$ for 5 min at room temperature prior to the addition of 9.4 μM F-actin. In one series of experiments, F-actin and UTR261 were incubated together prior to the addition of calmodulin and sedimentation. Following sedimentation (15 min, 426,000 × *g*, 4°C), equivalent volumes of the supernatant and pellet fractions were run on 15% SDS-polyacrylamide gels. Following staining and destaining, protein bands were analysed by volume integration on a Molecular Dynamics 300A computing densitometer.

UTR261- and calmodulin-Sepharose were prepared by coupling the respective purified proteins to CNBr-activated Sepharose (Pharmacia) according to the manufacturers instructions. Affinity chromatography of UTR261 on calmodulin-Sepharose, or calmodulin on UTR261-Sepharose, was carried out in 20 mM Tris-HCl, pH 8.0, 1 mM DTT (buffer A). UTR261 (1 mg) was loaded onto a calmodulin-Sepharose column in buffer A in the presence of 1 mM $CaCl_2$, following an additional wash with buffer A containing 1 mM $CaCl_2$ and 100 mM NaCl, the buffer was changed to buffer A containing 1 mM EGTA. Calmodulin (1 mg) was loaded onto a UTR261-Sepharose column as described above for UTR261. In one experiment UTR261 (1 mg) was loaded onto a calmodulin-Sepharose column in buffer A containing 1 mM EGTA and then washed with the same buffer containing 100 mM NaCl. Column fractions were run on 15% SDS-polyacrylamide gels and quantified by volume integration on a Molecular Dynamics 300A computing densitometer.

Potential calmodulin binding sites in the α-actinin, dystrophin and utrophin sequences were identified by the program described by Erickson-Viitanen and DeGrado [13]. Multiple sequence alignments were performed using Clustal V [14], and hydrophobicity and hydrophobic moments were calculated as previously described [15].

3. Results

We examined the ability of calmodulin to inhibit the binding of 19.6 μM UTR261 to F-actin (equivalent to half-maximal binding for this construct, K_d 19 μM [11]). In the absence of F-actin, UTR261 was recovered in the supernatant fraction;

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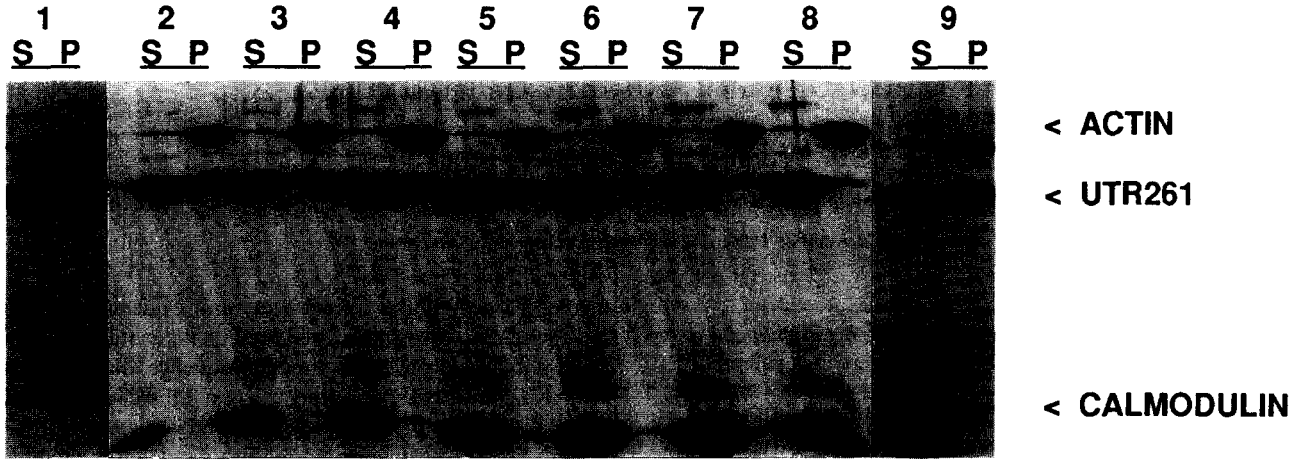

Fig. 1. Inhibition of utrophin binding to F-actin by Ca^{2+}/calmodulin. SDS-PAGE of supernatant (S) and pellet (P) fractions following sedimentation of 19.6 μM UTR261 with 9.4 μM actin and increasing concentrations of calmodulin from 0 μM (lanes 1) to 127 μM (lanes 8,9) either in the presence (lanes 1–8) or absence (lanes 9) of $CaCl_2$. The positions of actin, UTR261 and calmodulin are as indicated.

increasing concentrations of calmodulin inhibited the binding of UTR261 to F-actin but only in the presence of calcium (Fig. 1). The binding of UTR261 to F-actin was reduced 75% by 100 μM Ca^{2+}/calmodulin, whereas in the absence of calcium, calmodulin concentrations as high as 127 μM were unable to inhibit the binding of UTR261 to F-actin (Fig. 1, lanes 9; Fig. 2). This regulatory effect appeared to be specific for Ca^{2+}/calmodulin since the COOH-terminal Ca^{2+} binding domain of α-spectrin (itself a calmodulin-like structure) was unable to regulate UTR261–actin binding (S.J. Winder and G. Travé, unpublished observations). To determine the specificity of Ca^{2+}/calmodulin-dependent regulation of utrophin–actin binding we compared the highly related F-actin binding domains from dystrophin and α-actinin. Fig. 2 shows normalised data from densitometric scans of SDS-polyacrylamide gels of F-actin sedimentation assays performed with DMD246 and UTR261 and increasing concentrations of Ca^{2+}/calmodulin. Whilst both DMD246 and UTR261 were displaced from F-actin by calmodulin, at 150 μM calmodulin only ~40% of bound DMD246 was displaced from F-actin compared to ~75% of UTR261 at 100 μM calmodulin. AAC140 was displaced from F-actin to the same extent as DMD246 (data not shown). Half-maximal displacement of 19.6 μM utrophin from F-actin occurred at 37 μM calmodulin. Furthermore, the inhibition of AAC140 (not shown) and DMD246 binding to F-actin was not calcium-dependent. Regulation of UTR261 binding to F-actin, however, was 85% calcium-dependent at 127 μM calmodulin (Fig. 2), when compared to the EGTA control. In experiments where calmodulin was added to a preformed F-actin-UTR261 complex, the ability of calmodulin to inhibit F-actin binding was reduced from 75% (at 127 μM calmodulin) to 28%, but nevertheless remained completely calcium-dependent. Calmodulin is therefore able to regulate the F-actin–utrophin interaction in a calcium-dependent manner; by inhibiting the association of utrophin with F-actin and by disrupting the F-actin–utrophin complex.

We used affinity chromatography to test whether we could detect the calcium-dependent interaction between utrophin and calmodulin. When either calmodulin was applied to a UTR261-Sepharose column or UTR261 applied to a calmodulin-Sepharose column, approximately 10% of each protein was specifically and consistently retained at physiological salt concentrations and in the presence of calcium (Fig. 3). Binding of 100% of the respective ligand to the affinity column was not achievable due to the relatively low affinity of UTR261 for calmodulin. UTR261 applied to the calmodulin-Sepharose column in the absence of calcium (presence of 1 mM EGTA) was eluted from the column in the absence of added salt, and no further UTR261 was eluted when the buffer was changed to one containing 100 mM NaCl (Fig. 3). Similar results were obtained when calmodulin was applied to the UTR261-Sepharose

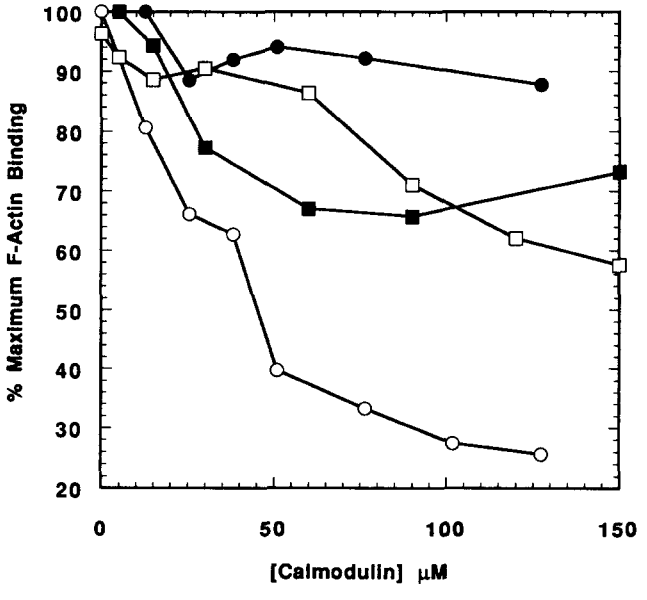

Fig. 2. Effect of calmodulin on utrophin and dystrophin binding to F-actin. Densitometric analysis of SDS-gels of UTR261 (●,○) and DMD246 (■,□) binding to F-actin in the presence of increasing concentrations of calmodulin and 1 mM $CaCl_2$ (open symbols) or 1 mM EGTA (filled symbols). Data are the mean of three separate experiments (standard errors of the mean were mostly less than 5% and have been omitted for clarity) and are presented as % maximum F-actin binding in order to normalise the data from UTR261 and DMD246 experiments.

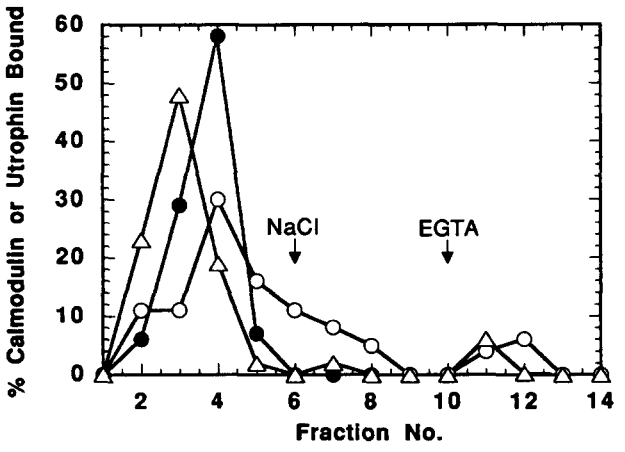

Fig. 3. Affinity chromatography of 1 mg of UTR261 (●,○) or calmodulin (△) on calmodulin-Sepharose or UTR261-Sepharose respectively, either in the presence (○,△) or absence (●) of calcium. Proteins were applied to the respective column in 20 mM Tris-HCl, pH 8.0, 1 mM DTT, with or without calcium, as indicated and fractions collected. At fraction 6 (arrow) the buffer was changed to one containing 100 mM NaCl, at fraction 10 (arrow) the buffer was changed to one containing EGTA (1 mM excess over calcium). Data are presented as percentages from densitometric scans of SDS-gels of the column fractions, as described in section 2.

column in the absence of calcium (data not shown). These data suggest a specific, though weak, calcium-dependent interaction between calmodulin and UTR261. Furthermore UTR261 was unable to inhibit the calmodulin-dependent activation of phosphodiesterase and did not increase the fluorescence of calmodulin-MIANS or calmodulin-DANS (S.J. Winder and J.D. Johnson, unpublished observations), all suggestive of a weak interaction between calmodulin and UTR261.

Analysis of the amino acid sequences of AAC140, DMD246 and UTR261 for putative calmodulin-binding basic amphiphilic α-helices, revealed a 15–16 residue region in all three proteins. In each case the predicted region was just NH_2-terminal of ABS2, the largest F-actin binding region in these proteins [11] (see alignment in Fig. 4). In AAC140 a region of 16 amino acids was predicted at low stringency to bind calmodulin, i.e. whilst meeting the general requirements for calmodulin binding [13] the aggregate mean hydrophobicity and hydrophobic moment for the 16 residue sequence was low. A similar region was also predicted to bind calmodulin in DMD246, however, while the average hydrophobicity for the 15 amino acid sequence was high (0.62), the aggregate hydrophobic moment was also low. The identical region in UTR261 (also 15 amino acids in length and homologous to the region in DMD246) had a high average hydrophobicity (0.61) and a considerably higher hydrophobic moment (1.92). These differences may explain why only utrophin–actin binding is regulated by calmodulin in a Ca^{2+}-dependent manner.

4. Discussion

α-Actinin is clearly an antiparallel homodimer [16] with F-actin bundling activity that can be regulated in a calcium-dependent way [1]. Whether the regulation is an inter- or intramolecular event is not clear, but given the juxtaposition of the calcium binding and F-actin binding regions in opposite molecules it is quite likely that the interaction is intermolecular. In single dystrophin and utrophin molecules, where the distance between the NH_2-terminal F-actin binding region and COOH-terminal calcium binding region is in the order of 110–175 nm [3,4], it is hard to envisage an intramolecular regulatory event occurring over such a distance: especially as the central rod domains of dystrophin and utrophin are predicted to be highly flexible [17]. It is likely that if such a regulatory event were to occur, it would be between the opposing ends of an antiparallel dimer (as in α-actinin and spectrin). The finding that the ubiquitous calcium binding protein, calmodulin, can regulate the F-actin binding activity of utrophin in a calcium-dependent manner suggests two things: firstly, there is regulation of the utrophin–actin interaction via a calmodulin-dependent mechanism. Secondly, the putative COOH-terminal calcium binding domain of utrophin may itself be able to regulate F-actin binding in the adjacent NH_2-terminal F-actin binding domain in an antiparallel utrophin dimer.

Several actin binding proteins have been shown to be regulated by Ca^{2+}-calmodulin; caldesmon [18], calponin [19], MAP1 and tau [20,21] and the heat shock proteins HSP90 and HSP100 [22,23] for example. It is therefore not inconceivable that the utrophin–actin interaction could also be regulated by calmodulin. Several members of the dystrophin associated glycoprotein complex have been reported to bind calmodulin [24], and dystrophin can be phosphorylated by a calmodulin-dependent kinase, probably Ca^{2+}/calmodulin-dependent kinase II [25]. It is not surprising, therefore, that utrophin, which is closely related to dystrophin, should also be regulated by Ca^{2+}-calmodulin. Given the low cellular concentration of utrophin (dystrophin is present in muscle at 0.002% of total cell protein [26]) and relatively high concentrations of calmodulin, the concentration of calmodulin required to regulate utrophin–actin binding (~2-fold molar excess to achieve 50% displacement) is easily obtainable within most cells. The organisation of the F-actin–utrophin–glycoprotein complex link can therefore be considered to be dynamic, with modulation by Ca^{2+}-calmodulin, thus allowing for the redistribution of utrophin in response to cell division, cell movement and external factors such as agrin which induces the clustering of acetylcholine receptors via the utrophin glycoprotein complex [27].

Intermolecular regulation of utrophin binding to F-actin has obvious implications for the higher order structural organisation of utrophin. In order for the COOH-terminus of one molecule to regulate the NH_2-terminus of another, they must be in close association, either as antiparallel side-by-side dimers or

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                                        ABS2
aac 78  LAKPERGKMRVHKISNVNKALDFIASKGVKLVSIGAEEIVDGNVKMTLGMIWTIILRFAIQDI 140
dmd 61  LPK-EKGSTRVHALNNVNKALRVLQNNNVDLVNIGSTDIVDGNHKLTLGLIWNIILHWQVKNV 124
utr 79  LPK-ERGSTRVHALNNVNRVLQVLHQNNVELVNIGGTDIVDGNHKLTLGLLWSIILHWQVKDV 140
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Fig. 4. Identification of predicted calmodulin binding regions in α-actinin, dystrophin and utrophin. Part of a Clustal V alignment of the NH_2-terminal F-actin binding regions of α-actinin, (aac); dystrophin (dmd) and utrophin (utr), residue numbers are indicated at each end of the sequences. The principal F-actin binding site, ABS2 [11] is shown boxed and the predicted calmodulin binding regions referred to in the text are in bold and underlined.

end-to-end dimers. Whilst no electron micrographs of utrophin are available, dystrophin has been shown to self-associate both as side-by-side and end-to-end dimers [3,4] and even as tetramers and octamers [4]. At present, however, there is no evidence to indicate whether the side-by-side dimers are parallel or antiparallel.

The finding that the binding of utrophin and dystrophin to F-actin are regulated to different extents by Ca^{2+}/calmodulin suggests that the over-expression of utrophin as a potential therapy for sufferers of Duchenne's and Becker's muscular dystrophy may be over-simplified. Further work is necessary to establish the functional properties of these two highly related proteins before such an approach could be considered. Production of the COOH-terminus of utrophin (and dystrophin) in bacteria, analysis of its calcium binding properties and effects on the binding of the NH_2-terminus of utrophin to F-actin will help resolve some of these questions.

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Note added in proof

In a recent article Bonet-Kerrache et al. [(1994) FEBS Lett. 355, 49–53] have shown that bacterially expressed N-terminal domains of dystrophin bind calmodulin in a calcium-dependent manner, however, as we have found, calmodulin did not directly regulate the binding of dystrophin to actin.