Disruption of the utrophin–actin interaction by monoclonal antibodies and prediction of an actin-binding surface of utrophin

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Monoclonal antibody (mAb) binding sites in the N-terminal actin-binding domain of utrophin have been identified using phage-displayed peptide libraries, and the mAbs have been used to probe functional regions of utrophin involved in actin binding. mAbs were characterized for their ability to interact with the utrophin actin-binding domain and to affect actin binding to utrophin in sedimentation assays. One of these antibodies was able to inhibit utrophin–F-actin binding and was shown to recognize a predicted helical region at residues 13–22 of utrophin, close to a previously predicted actin-binding site. Two other

mAbs which did not affect actin binding recognized predicted loops in the second calponin homology domain of the utrophin actin-binding domain. Using the known three-dimensional structure of the homologous actin-binding domain of fimbrin, these results have enabled us to determine the likely orientation of the utrophin actin-binding domain with respect to the actin filament.

Key words: calponin homology domain, dystrophin, epitope mapping, fimbrin, phage display.

INTRODUCTION

Utrophin [1], the autosomal homologue of dystrophin, is a member of the dystrophin/actinin/spectrin class of actin-binding proteins, each of which contain an N-terminal actin-binding domain comprising two repeating sequences [2], termed calponin homology (CH) domains [3–5]. In utrophin and dystrophin, these actin-binding domains serve as simple intracellular anchors to the F-actin cytoskeleton, and do not play any role in organizing actin filaments or regulating their assembly or disassembly [6,7]. A number of studies investigating the actin-binding regions of dystrophin, utrophin, α -actinin and β -spectrin have implicated three regions of the primary sequence as being crucial for actin binding [7]. While distant in the primary sequence, these three actin-binding sites (ABSs; see Figure 4A), which are largely basic and hydrophobic in nature, were assumed to come together as a single actin-binding region in the tertiary structure of this class of domain [8,9]. The recently elucidated crystal structure of the first actin-binding domain of the more distantly related actin bundling protein, fimbrin, however, has proved otherwise [10]. The three ABSs in fimbrin are not only well separated in the tertiary structure, but the majority of the basic and hydrophobic residues in these regions are buried within the core of the structure and are not available to interact with actin [10]. A large body of data obtained using a variety of methods (NMR, peptides, and point, deletion and truncation mutagenesis) has failed to produce a consensus as to the precise determinants for actin binding in utrophin and dystrophin (see [9] for a review). As shown previously for α -actinin [11], β -spectrin [4] and dystrophin and utrophin [7,9], the second (C-terminal) CH domains of these actin-binding proteins do not have the intrinsic ability to bind to actin. Nevertheless, they are clearly involved in contributing to the affinity of the N-terminal CH domain for F-actin, such that the complete actin-binding domain comprising two CH domains

together has a considerably higher affinity for F-actin than the N-terminal CH domain alone.

We have previously generated a panel of 15 monoclonal antibodies (mAbs) against utrophin using residues 113–371 (the second CH domain and part of the triple-helical coiled-coil repeat) of human utrophin [12]. These, together with four new mAbs against residues 1–112 (the N-terminal CH domain), have now been characterized and evaluated for their ability to inhibit F-actin binding to utrophin. Very precise mapping of mAb-binding sites on proteins is sometimes possible using phage-displayed peptide libraries. This approach to epitope mapping often allows assessment of the importance of individual amino acids within the epitope. Binding studies with mapped mAbs have been used to predict dystrophin/utrophin structure and folding, using the general principle that epitope residues must be accessible on the antigen surface for mAb binding to occur [13,14]. We now show that mapped mAbs can provide a novel approach to understanding utrophin–actin interaction at the molecular level by studies of their ability to prevent and disrupt this interaction.

MATERIALS AND METHODS

Utrophin expression constructs

Utrophin constructs comprising residues 1–112 and 113–371 were prepared as described previously [7]. Utrophin residues 1–261 and 28–261 were generated by PCR using standard cloning techniques utilizing 5' primers containing an in-frame *NdeI* site and 3' primers containing a stop codon and a *SalI* site. *NdeI*- and *SalI*-restricted PCR fragments were ligated into a similarly digested pSJW1 vector [15,16]. Expression constructs were sequenced to check for PCR-generated errors, and recombinant

Abbreviations used: ABS, actin-binding site; CH, calponin homology; mAb, monoclonal antibody; UTR261, utrophin-(1–261).

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proteins were expressed in *Escherichia coli* BL21(DE3) and purified from the supernatant of bacterial lysates as described previously [7].

mAbs and epitope mapping

Fifteen mAbs (MANNUT1–MANNUT15), produced by immunization of Balb/c mice with residues 113–371 of utrophin and fusion of spleen cells with Sp2/O myeloma cells, have been described previously [12]. Four further mAbs, MANNUT16 (4H12), MANNUT17 (6H7), MANNUT18 (7C7) and MANNUT19 (8B5), were produced by immunization with residues 1–261 of utrophin. These four mAbs were screened by ELISA to confirm binding to residues 1–261, but not to residues 113–371. All mAbs were selected for their ability to recognize utrophin, but not dystrophin, on Western blots and on human muscle sections [12]. Hybridoma fusion and selection methods have been described in detail elsewhere [17]. A phage-displayed 15-mer peptide library in the pIII protein of fuse5 filamentous phage [18] was generously donated by George P. Smith (University of Missouri, Columbia, MO, U.S.A.), and was screened as described previously [19].

Actin binding

F-actin was prepared from rabbit skeletal muscle acetone powder and F-actin sedimentation assays were performed as previously described [7]. F-actin binding was assessed by sedimentation in a Beckman TL100 benchtop ultracentrifuge (15 min, 426000 g, 4 °C), using approx. 12 μ M F-actin in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol and 1 mM EGTA (actin binding buffer). Equal quantities of supernatant and pellet fractions were quantified by volume integration of 15% (w/v) acrylamide mini-SDS/polyacrylamide gels on a Molecular Dynamics 300A scanning densitometer. The amount of protein bound to actin was calculated from the known concentrations of proteins in the assay and the ratios of each protein in the supernatant and pellet, with corrections for trapping (1.4%), as described previously [6,11]. Up to 70% of the total assay volume (100 μ l) was taken up with mAb culture supernatant or a 1:1 (v/v) dilution of ascites in PBS. Where mAbs were added to utrophin prior to or after the addition of actin, the antibody/utrophin or utrophin/F-actin mixture was incubated for 5 min at room temperature before the addition of F-actin or mAb respectively, and was then incubated for a further 5 min before centrifugation as above.

RESULTS

Actin binding

Initial screens of 19 mAbs and four polyclonal sera for their ability to inhibit utrophin-(1–261) (UTR261) binding to actin were carried out in triplicate in the presence of 2 μ M UTR261, 12 μ M F-actin and a constant 70% of the total assay volume representing hybridoma culture supernatant or 20% (v/v) serum. The presence of up to 70% tissue culture medium or 20% foetal-calf, mouse, rabbit or horse serum in the actin binding assay had no effect on UTR261 binding to F-actin (results not shown). Culture supernatants were also tested for their ability to immunoprecipitate UTR261 alone under actin-binding assay conditions, but in the absence of F-actin. None of the culture supernatants were able to immunoprecipitate UTR261 under these conditions. Culture supernatants that gave a consistent change in UTR261 binding to F-actin, as judged by both a decrease/increase in UTR261 in the pellet fraction and a corresponding increase/

Table 1 Characteristics of mAbs against the utrophin N-terminus

Epitopes are those deduced from epitope mapping studies described in Figure 3. Upper-case letters represent reproducible matches with the utrophin primary sequence, lower-case letters indicate occasional matches, and x represents no significant match between peptide and utrophin sequence. The 'sequence' column gives the start and finish residue numbers of the deduced epitope in the utrophin sequence.

Antibody	Residues comprising antigen	Epitope	Sequence
MANNUT3	113–371	PDxFxWD	194–200
MANNUT4	113–371	PDLfxWD	194–200
MANNUT8, 12, 13	113–371	TLpxxY	265–270
MANNUT16	1–261	gQxxFSxxl	13–22

Figure 1 Inhibition of utrophin binding to F-actin by mAb MANNUT16 (A, B), and Western blotting of utrophin-(28–261) and UTR261 by MANNUT16 (C)

F-actin (12 μ M) was incubated with increasing concentrations of UTR261. Lanes 1–6 are the pellet fractions only of F-actin incubated in the presence of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 μ M UTR261 respectively without (A) or with (B) a 40-fold dilution of MANNUT16 ascites equivalent to 0.5 μ M IgG. Data are representative of two independent experiments. Actin and utrophin bands are as indicated; other constant bands on the gels are derived from the culture supernatants added to all samples. (C) Western blotting of 2 μ g of utrophin-(28–261) (UTR28–261, lane 1) and UTR261 (lane 2) by a 1:10 dilution of MANNUT16 in PBS.

decrease in UTR261 in the supernatant fraction, were examined further over a range of dilutions of culture supernatants and UTR261 concentrations. None of the polyclonal sera (one raised in rabbit and three in mice) had any effect on actin binding. Of the 19 mAbs tested, six (MANNUT3, 4, 9, 12, 14 and 16; see Table 1) were analysed further as described in the Materials and methods section. Of these six, MANNUT16, raised against residues 1–261 of utrophin, gave the strongest inhibition of actin binding (Figures 1A and 1B; Figure 2) when compared both with other mAbs raised against utrophin and with an unrelated control antibody, CK-JAC [20], raised against creatine kinase (Figure 2, and results not shown). Whether added to utrophin before incubation with F-actin or to a preformed complex of utrophin–F-actin, MANNUT16 was able to inhibit the binding of utrophin to F-actin (Figure 2).

Epitope mapping

Twelve mAbs (MANNUT2–MANNUT13 inclusive) were pooled and used to select 15-mer peptides from a phage-displayed library by biopanning. Screening results with these mAbs are shown in detail in [19]. A total of 21 positive phage clones obtained after two rounds of biopanning were recognized by MANNUT4 or MANNUT3. When the library was

Figure 2 Effects of mAbs on the binding of utrophin to F-actin

MANNUT16 was added to the indicated concentrations of utrophin, before (■) or after (□) the addition of 10 μ M F-actin, followed by centrifugation, SDS/PAGE of pellet fractions and densitometric analysis as described in the Materials and methods section. MANNUT16 prevents the increase in utrophin binding observed in the presence of control mAbs MANNUT17, MANNUT18 or MANNUT19, or in the absence of antibody.

Antibody	Peptide Sequence	
MANNUT8, 12, 13	TLPPRYPSPSRSHHIDD	
MANNUT8, 12, 13	TLPPRYPSPASHHIDD	
Utrophin:	TLPRKYKKECEEAT	265–280
MANNUT3, 4	AGGF P DLIFWDHYYS	
MANNUT3, 4	AGGI P DLYFWDHIYS	
MANNUT3, 4	LFDFDSSDSTGCMC	
MANNUT3	VPDKFCWDLRGCGA	
Utrophin:	HRHK P DLF S WDKVVKMSPIE	189–205
MANNUT16	QASFS A FLGGVPAPFS	
MANNUT16	GGQDA F SWLIASASV	
MANNUT16	SQRSF S VVISALRGDA	
Utrophin:	NGQNE F SDI I KSRSE	13–28

Figure 3 Epitope mapping of anti-utrophin mAbs

Positive peptides from the random 15-mer peptide library are shown along with the mAbs recognizing them. Although a larger number of phage clones were selected and sequenced, many of these expressed the same peptide sequences. At the bottom of each section, the corresponding region of utrophin that matches the detected epitope is shown, with common amino acids in bold. The corresponding residue numbers of the utrophin sequence are to the right, and the deduced epitope in the utrophin sequence is underlined.

screened again using the mAb mixture without MANNUT4, 17 phage clones were recognized by MANNUT3 and a further 29 clones were recognized by MANNUT8, MANNUT12 and MANNUT13. The library was screened for a third time using the remaining seven mAbs alone, but no further phage clones were

selected. The library was also screened using MANNUT16 alone, and three positive phage clones were obtained.

The results from sequencing the positive phage clones are shown in Figure 3. MANNUT8, MANNUT12 and MANNUT13 recognized the sequence TLPxxY (where x represents no significant match between peptide and utrophin sequence) (Figure 3), corresponding to utrophin-(265–270), outside of the recognized actin-binding domain [7]. The sequential epitope recognized by MANNUT3 and MANNUT4 is PDLF**x**WD (Figure 3), corresponding to utrophin-(193–199). Leu-195 is essential for MANNUT4 binding (Figure 3). Although it is within the actin-binding domain of utrophin, this epitope is restricted to the second CH domain, which is probably not directly involved in actin binding. Indeed, this epitope maps to a variable surface loop structure between conserved helices 2 and 3 in the second CH domain (N. H. Keep, F. L. M. Norwood, C. A. Moores, S. J. Winder and J. Kendrick-Jones, unpublished work) and would not be predicted to affect actin binding. The MANNUT16 epitope was mapped to the extreme N-terminus of utrophin, i.e. residues 13–22. The location of this epitope to the N-terminal 28 residues of utrophin was also confirmed by Western blotting (Figure 1C); MANNUT16 did not detect a utrophin construct comprising residues 28–261 of utrophin (Figure 1C, lane 1), whereas it did detect UTR261 (Figure 1C, lane 2). Residues 13–22 of utrophin are N-terminal to the first CH domain, but in utrophin, unlike in the closely related proteins spectrin and fimbrin (whose structure is known), this region is predicted to form an extended helix N-terminal to the structurally important ABS1 [7,10]. A helical conformation is also suggested by the distribution of the amino acids within the mapped epitope, QXXFSXXI, consistent with antibody binding to one face of the helix [14,21].

DISCUSSION

The use of mAbs to dissect functional and structural aspects of protein–protein interactions has its limitations; antibodies are large entities, and any apparent effect against a discrete epitope may be due to steric hindrance over a much greater distance. Nevertheless, antibodies do point to surface features of proteins and, where they do not interfere with a particular protein–protein interaction, it can be reasonably predicted that these regions are not near, or involved in, the site(s) of interaction. Coupled with a knowledge of protein structure, either actual or predicted, they become powerful tools in determining the importance of motifs and structural features involved in protein function. Most epitopes on native ‘globular’ proteins, like the utrophin N-terminus, are likely to be conformational, though some may have a ‘core’ sequence that can be mimicked sufficiently by peptides for antibody binding to occur. It is possible, therefore, that the utrophin sequences identified in the present study using phage-displayed peptides do not represent the full extent of the epitope, although they do serve to locate the epitope accurately at the utrophin surface.

The MANNUT12 epitope was mapped to residues 265–270 of utrophin, i.e. outside the part of the protein comprising residues 1–261 that is considered to be the actin-binding domain [1,7], so it is not surprising that MANNUT12 had no effect on actin binding of UTR261. From the structure of the second utrophin CH domain (N. H. Keep, F. L. M. Norwood, C. A. Moores, S. J. Winder and J. Kendrick-Jones, unpublished work), and by analogy with the published structures of the homologous regions of fimbrin [10] and β -spectrin [4], the epitope recognized by MANNUT3 and MANNUT4 (residues 193–199) forms part of an extended loop structure on the surface of the C-terminal CH

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