Cloning and expression of full length mouse utrophin: the differential association of utrophin and dystrophin with AChR clusters

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Abstract We have cloned and sequenced mouse utrophin cDNA, and successfully expressed full length utrophin (400 kDa) in both muscle and non-muscle cells. The expression of recombinant utrophin is compared with that of its homologue, dystrophin (427 kDa). We demonstrate that recombinant utrophin is targeted into agrin-induced acetylcholine receptor (AChR) clusters, while recombinant dystrophin is evenly distributed along cell membranes in cultured Sol 8 muscle cells. This observation suggests that utrophin and dystrophin may interact with different cytoskeletal proteins. The C-terminal domains are found to be responsible for the association of utrophin with AChR clusters.

Key words: Utrophin; Dystrophin; Acetylcholine receptor; Neuromuscular junction; cDNA cloning; Gene expression

1. Introduction

Dystrophin is part of an elaborate protein complex that bridges the inner cytoskeleton and the extracellular matrix [1]. In the X-chromosome-linked Becker and Duchenne muscular dystrophy [2], mutations in dystrophin disrupt the integrity of the complex, leading to fragility in muscle membrane and muscle necrosis. Utrophin, the discovered autosomal homologue of dystrophin, shares considerable sequence homology with dystrophin [3]. In addition to structural similarities, utrophin and dystrophin are also found to be associated with identical or similar proteins [4,5]. In both DMD patients and *mdx* mice where dystrophin is absent, utrophin is upregulated [6]. These observations lead to the suggestion that these two proteins may have similar function(s), raising the hope that still higher levels of utrophin could ameliorate symptoms of muscular dystrophy by substituting for dystrophin [7]. On the other hand, utrophin and dystrophin have different tissue distributions and are differentially regulated during development [3,8]. At the adult neuromuscular junction, utrophin is also found to be exclusively localized with nerve-induced AChR clusters while dystrophin is enriched at the junction [6]. The above observations suggest that, despite structural similarities, there may be substantial functional differences between utrophin and dystrophin, e.g. at the neuromuscular junction. As a beginning step to studying the structural and functional differences between utrophin and dystrophin, we have cloned, sequenced, and expressed the full length mouse recombinant utrophin. We demonstrate that recombinant utrophin is targeted into agrin-induced AChR clusters while recombinant dystrophin is evenly distributed along cell membranes in cultured Sol 8 cells. We also show that the C-terminus of utrophin is responsible for targeting recombinant utrophin into agrin-induced AChR clusters.

2. Materials and methods

2.1. cDNA library, cloning and sequencing

A 1.9 kb human C-terminus utrophin cDNA fragment [9] obtained from ATCC was used as a probe to screen an oligo dT-primed cDNA library constructed using mRNA from a BC3H1 cell line. Approximately 3 kb of C-terminal mouse utrophin was isolated and used in further screening of a randomly primed cDNA library constructed using mRNA from adult mouse lung (Stratagene, La Jolla, CA). Library screening, plaque purification, and DNA isolation were performed as described previously [10]. All fragments of mouse utrophin cDNA obtained from library screening were subcloned into Bluescript KS II$^+$ vectors (Stratagene) and further ligated together into a single continuous 11 kb coding sequence. DNA sequencing was performed both manually using designed oligonucleotides and with an automated sequencer using a Taq Dyedeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster city, CA). The sequence was determined on both strands.

2.2. Expression constructs

The 11 kb full length mouse utrophin cDNA was engineered at the 5′-end with a Flag epitope tag by special oligo design and subcloned into a Bluescript KS II$^+$ vector containing the cytomegalovirus (CMV) promoter and SV40 poly A tail (Fig. 2). The deletion mutant, MU-1 (Fig. 4), was generated by subcloning a cDNA fragment containing a stop codon at nucleotide 3416 of the full length mouse utrophin into the same expression vector. MU-2 and MU-3 were generated by specific restriction enzyme digestion, and subcloned into expression constructs. Each of the three mutants was tagged with Flag epitope after the first initiation codon, methionine. The dystrophin expression construct was pMDA, which contained the full length murine dystrophin cDNA regulated by 6.5 kb MCK promoter/enhancer/first intron fragment [11].

2.3. Maintenance and transfection of cells

QT-6 cells and Sol 8 cells were maintained and transfected as previously described [12,13]. Agrin-secreted CHO cells, gifts from R. Scheller, were cultured as described previously [14]. The recombinant agrin was concentrated from agrin-secreted culture medium using Centriprep-30 (Amicon Inc., MA).

2.4. Immunofluorescence and Western blotting

Fused myotubes were treated with the isolated form of agrin from CHO cells for 12–14 h, followed by incubation with rhodamine-labeled α-bungarotoxin (BGT) at 37°C for 1 h. These cells were then fixed, permeablized and blocked as described previously [12]. After blocking, each glass coverslip was taken out and incubated with the desired primary antibody, as detailed below. Anti-Flag antibody (1:50) was from Eastman Kodak Company, NY; anti-C-terminus of utrophin (1:30) was generated by the authors against a peptide containing the last 10 amino acids; anti-C-terminus of dystrophin (1:8) was obtained from Novocastra, UK (DYS-2). Following primary antibody treatment, the cells were incubated with the FITC- or TRITC-conjugated secondary antibody (Boehringer Mannheim

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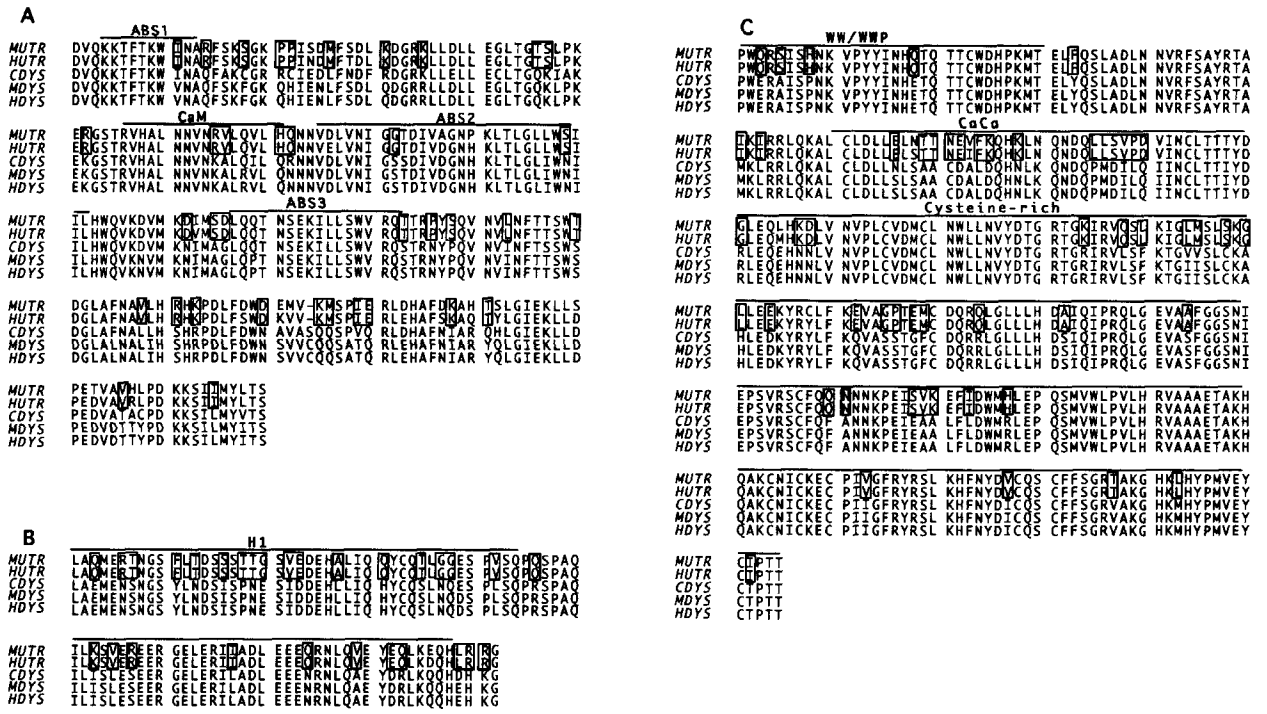

Fig. 1. Sequence comparison of the actin (A), the syntrophin (B) and the β-dystroglycan (C) binding domains of mouse utrophin (MUTR) with those of human utrophin (HUTR), and chicken (CDYS), mouse (MDYS), and human (HDYS) dystrophins. The boxed areas indicate residues that differ between utrophin and dystrophin. ABS1–3: major actin binding regions; CaM: a putative calmodulin binding site; CaCa: calcium binding EF-hands. The inset is a schematic representation of utrophin showing the location of domains A, B, and C.

Corp., IN). For Western blotting, proteins from membrane fractions isolated from both transfected and nontransfected cells were separated on 6% SDS PAGE gel, transferred onto a nitrocellulose sheet, blocked with 5% nonfat milk and incubated with a primary antibody (anti-Flag: 1–1000; anti-C-terminus of utrophin: 1–500; anti-C-terminus of dystrophin: 1–30), followed by a peroxidase-conjugated secondary antibody. Protein bands were visualized using the ECL method (DuPont, MA).

3. Results and discussion

3.1. Cloning and sequencing of full length mouse utrophin

Before this study, only the primary sequence of the human utrophin was known. Since much could be learned from the comparison of a new sequence to known ones of other related proteins, we set out to clone the cDNA that encoded the entire open reading frame of mouse utrophin. This was achieved through extensive library screening. Parts of C-terminal mouse utrophin cDNA from both the BC3H1 library and the mouse lung library were sequenced to verify that there was no polymorphism among these tissues. The complete sequence of the full length mouse utrophin cDNA was subsequently obtained. The availability of mouse utrophin sequence permitted the comparison of utrophins (human and mouse) with dystrophins (human, mouse and chicken), particularly the identification of utrophin sequences that are conserved between species, but differ from dystrophin. This may also assist the identification of functional domain(s) of utrophin through mutagenesis.

Fig. 1 compares the sequences of potentially important domains of utrophins and dystrophins. Like its orthologue and homologue, mouse utrophin contains an actin binding domain, a long region containing multiple repeats followed by a WW/WWP domain [4], a calcium binding EF-hand domain, a cysteine-rich domain, and two helical domains at the C-terminus. Mouse utrophin shares 84% identity at the nucleotide level and 87% identity at the amino acid level with human utrophin. Sequences at both ends of the molecule are particularly well conserved between the two species: there was 92% identity for the first 250 amino acids at the N-terminus; the best conserved region is found for residues starting from the WW/WWP domain through the C-terminus, with 97% identity, pointing to the potential functional significance of this region. The same regions of utrophin also show 70–80% identity to those of dystrophin. Within the actin binding domain (Fig. 1A) [4], the syntrophin binding domain (Fig. 1B) [15], and the β-dystroglycan binding region (Fig. 1C) [16], there are some amino acid residues which are nearly identical between mouse and human utrophins but are quite different from those in the dystrophins. Perhaps, these small differences in the primary structure could lead to behavioral differences between utrophins and dystrophins, e.g. in actin binding regulated by calmodulin [4]. Such small differences in the primary structure could also be responsible for differences in their roles in AChR clustering, as shown below.

3.2. Expression of mouse utrophin

For functional analysis, we ligated partial mouse utrophin cDNAs to form an 11 kb full length cDNA (10.3 kb coding sequence). This full length cDNA was tagged, subcloned into an expression vector containing the CMV promoter (Fig. 2), and transiently transfected into QT-6 fibroblasts. Membrane fractions were isolated and subjected to Western blot detection using either an anti-Flag antibody (1 and 2, Fig. 2A) or an antibody against the last 10 amino acids of the C-terminus

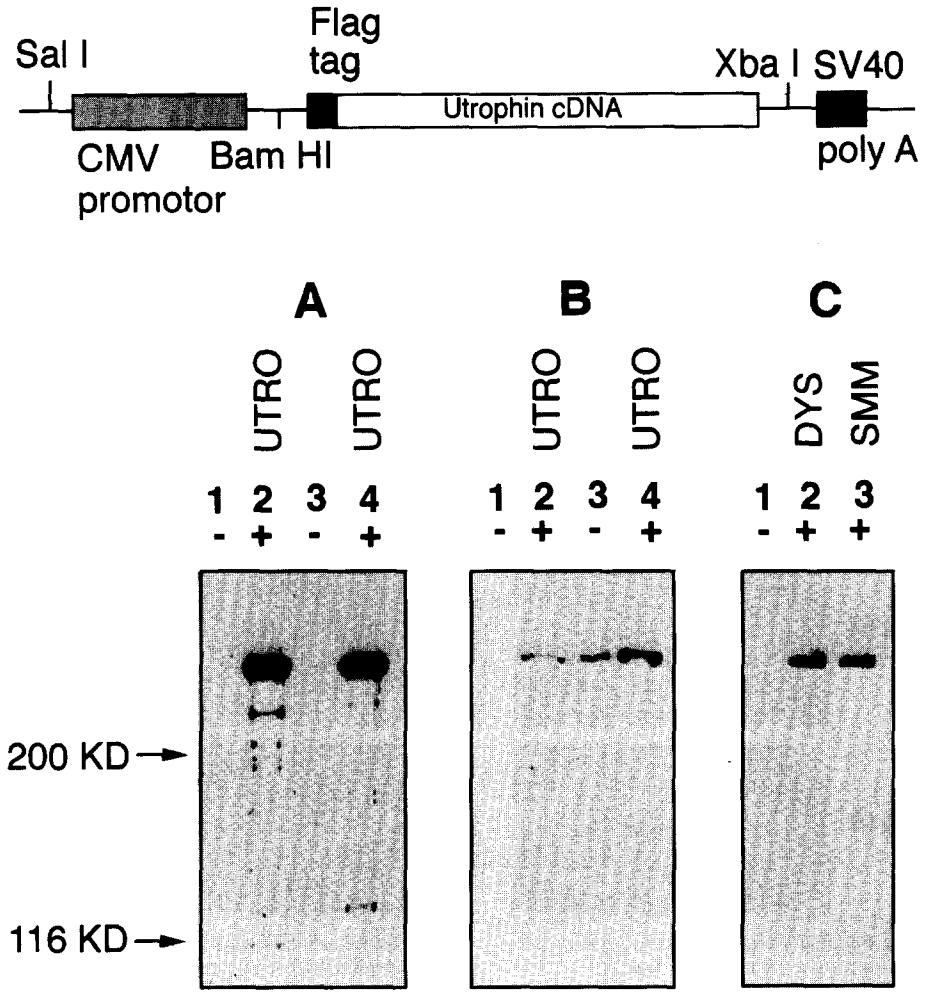

Fig. 2. Expression of recombinant utrophin and dystrophin in cultured cells. Top: Schematic representation of the utrophin expression construct. Bottom: Immunoblotting of utrophin expression in (A) QT-6 and (B) Sol 8 cells. In both cell lines, an anti-Flag antibody (2) and a polyclonal antibody against the last 10 amino acids of the C-terminus of human utrophin (4) were used for detection; lanes 1 and 3 were nontransfected cells used as negative controls for 2 and 4, respectively. Dystrophin expression (C) in Sol 8 cells was detected using a monoclonal antibody against the last 17 amino acids of human dystrophin (2). Nontransfected cells were used as negative control (1). A skeletal muscle membrane fraction was used as positive control (3).

(3 and 4, Fig. 2A). Both antibodies recognized a protein of 400 kDa. There was no detectable protein band around 400 kDa in nontransfected cells.

While the expression of full length recombinant utrophin was successfully demonstrated using QT-6 cells, this cell line was not suitable for further functional analysis, due to the lack of necessary components for producing agrin-induced receptor clusters. Instead, we chose Sol 8 cells, a cell line derived from the mouse soleus muscle and used previously in studying the regulation of AChR subunit expression [13]. Membrane fractions from nontransfected and transfected Sol 8 cells were isolated and proteins were detected by an anti-Flag (lanes 1 and 2, Fig. 2B) and an anti-C-terminal utrophin antibody (lanes 3 and 4, Fig. 2B). The anti-C-terminus antibody not only recognized recombinant utrophin (lane 4, Fig. 2B), as demonstrated by the elevated protein density for the same amount of protein loading, but also recognized endogenous utrophin (lane 3, Fig. 2B). Both recombinant and endogenous utrophins detected by the anti-C-terminus antibody have the same molecular weight as the protein recognized by the anti-Flag antibody (lane 2, Fig. 2B). These results demonstrate the successful expression of recombinant utrophin in Sol 8 cells, albeit with much lower efficiency than in QT-6 cells.

For comparison, the expression of full length recombinant dystrophin was also examined in Sol 8 cells (Fig. 2C). A mouse skeletal muscle membrane fraction was used as positive control. There was no detectable endogenous dystrophin in Sol 8 cells (lane 1, Fig. 2C). In Western blots of membrane proteins from Sol 8 cells transiently transfected with dystrophin cDNA, the expressed protein migrated at the same position as that detected in the skeletal muscle membrane fraction.

3.3. *Association of recombinant utrophin and dystrophin with agrin-induced AChR clusters*

The successful expression of full length mouse utrophin allows us to analyze functional similarities and differences between utrophin and dystrophin. We choose to assess the association of utrophin and dystrophin with agrin-induced AChR clusters. Although the exact mechanism by which agrin-induced AChR clustering is not clear, recent studies suggest that agrin released by the nerve can induce molecular reorganization involving AChR clusters at the motor endplate via a MuSK receptor complex [17,18]. It is speculated that

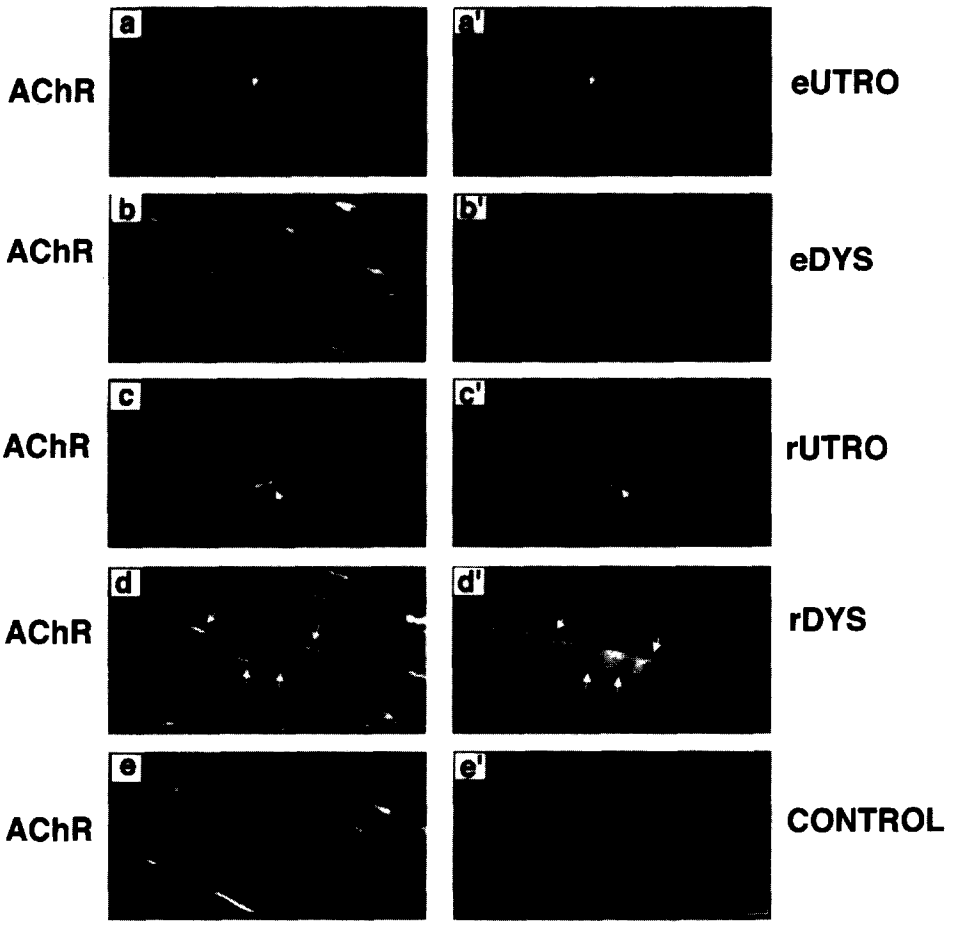

Fig. 3. Colocalization of recombinant utrophin but not dystrophin with agrin-induced AChR clusters in Sol 8 cells. Panels on the left (a–e) are staining for receptors. Panels on the right are staining for: (a′) endogenous utrophin; (b′) endogenous dystrophin; (c′) recombinant utrophin; (d′) recombinant dystrophin; and (e′) control experiment with no primary antibody. Instead of using the antibody against the C-terminus for endogenous utrophin (a′), an anti-Flag antibody was used as the primary antibody for recombinant utrophin (c′). A monoclonal antibody against the last 17 amino acids of human dystrophin was used for dystrophin (b′ and d′). Bar: 40 μm.

utrophin may act as a structural linker in connecting rapsyn-initiated small clusters to form large receptor clusters in responding to the binding of agrin to its receptor complex [12].

Because AChR clustering had never been documented in Sol 8 cells, we first used immunostaining to show that Sol 8 cells can produce both spontaneously formed and agrin-induced AChR clusters (Fig. 3). A comparison with previous studies showed that the number of spontaneously formed clusters in Sol 8 was much lower than that produced in C2 myotubes [12]. However, the number of AChR clusters in Sol 8 cells could be drastically increased by agrin, from 1–2 per field (420 μm × 420 μm) to 40 ± 5 per field. Rapsyn (data not shown) and utrophin both colocalized with all clusters (Fig. 3a′). Cluster length ranged from 6 to 70 μm. Consistent with Western blot analysis (Fig. 2C), immunofluorescence staining showed no detectable endogenous dystrophin (Fig. 3b′).

To determine whether recombinant utrophin is targeted into agrin-induced large receptor clusters, we transfected the Flag-tagged utrophin construct into Sol 8 cells. As shown by immunostaining, recombinant utrophin was colocalized with agrin-induced large receptor clusters (Fig. 3c′). Control experiments (secondary antibody alone) ruled out the possibility that the staining of clusters resulted from bleeding through from the receptor channel (Fig. 3e′).

We have also transfected recombinant dystrophin into Sol 8 cells. Recombinant dystrophin was evenly associated with plasma membrane and not colocalized with AChR clusters (Fig. 3d′), regardless of the level of expression. Thus our results have demonstrated that recombinant utrophin and dystrophin show differential association with AChR clusters in Sol 8 cells, providing evidence that it is utrophin and its complex that are specifically associated with AChR clusters. This association may result from the interaction of utrophin with synapse specific protein(s), such as AChR itself, rapsyn, syntrophin or other unidentified protein(s). Meanwhile, dystrophin may play a role in the maintenance of muscle membrane stability by interacting with different protein(s). Support for this hypothesis can be found in the observation that anti-β_2 syntrophin staining was only present at NMJ in rat skeletal muscle, while anti-α_1 syntrophin, an isoform of β_2 syntrophin, gave strong labeling of both sarcolemma and NMJ in normal rat and mouse muscle, resembling the localization of dystrophin [19]. In addition, anti-α_1 syntrophin gave similar but much weaker labeling in mdx mouse muscle where dystrophin was absent. Therefore, β_2 syntrophin appears to be specific to the NMJ, as is utrophin, while α_1 syntrophin is more likely associated with dystrophin.

The successful expression of functional full length mouse recombinant utrophin also enables future research on revealing possible functional differences between utrophin and dys-

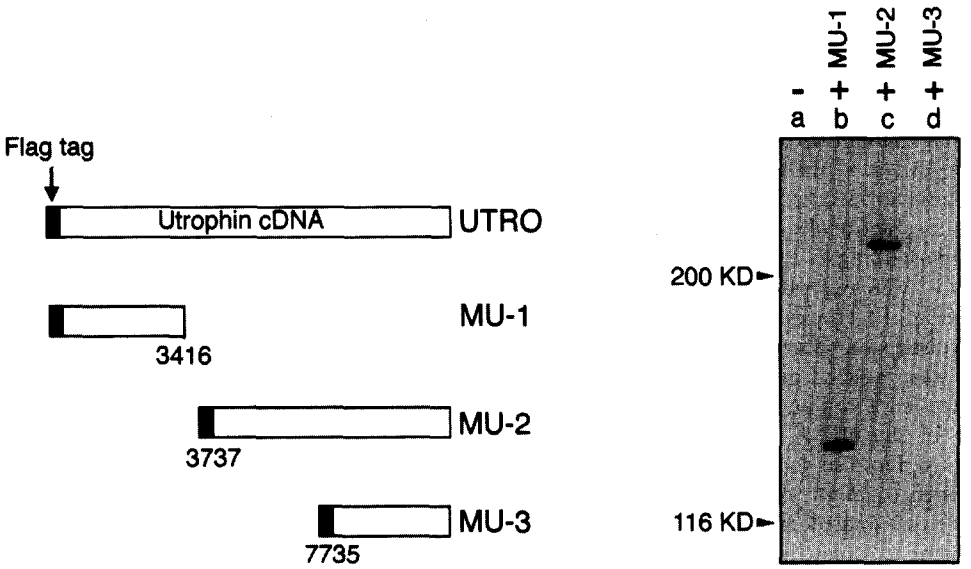

Fig. 4. Expression of utrophin mutants. Left: Schematic representations of expression constructs of wild type utrophin (WU) and utrophin mutants (MU-1, MU-2 and MU-3). Right: Immunoblotting of MU-1 (b), MU-2 (c) and MU-3 (d) expression in Sol 8 cells. Membrane proteins from nontransfected cells were used as negative control (a). An anti-Flag antibody was used for mutant expression detection.

trophin beyond the NMJ [20]. Previous studies indicate that utrophin expression is upregulated in both *mdx* mice and DMD patients where dystrophin is absent, suggesting that utrophin may compensate for the loss of dystrophin. On the other hand, the binding of utrophin and dystrophin to F-actin are regulated to different extents by Ca^{2+}/calmodulin [4]. Thus the proposal of using overexpression of utrophin as a potential therapy for DMD patients may be over-simplified. In this regard, the availability of full length functional utrophin expression construct may allow us in the near future to systematically address this important issue.

3.4. *C-terminal domains are responsible for targeting recombinant utrophin into AChR clusters*

To understand at the molecular level such differential localization of utrophin and dystrophin with AChR clusters, we generated three utrophin deletion mutants (Fig. 4). MU-1 contained N-terminal domain and small portion of central

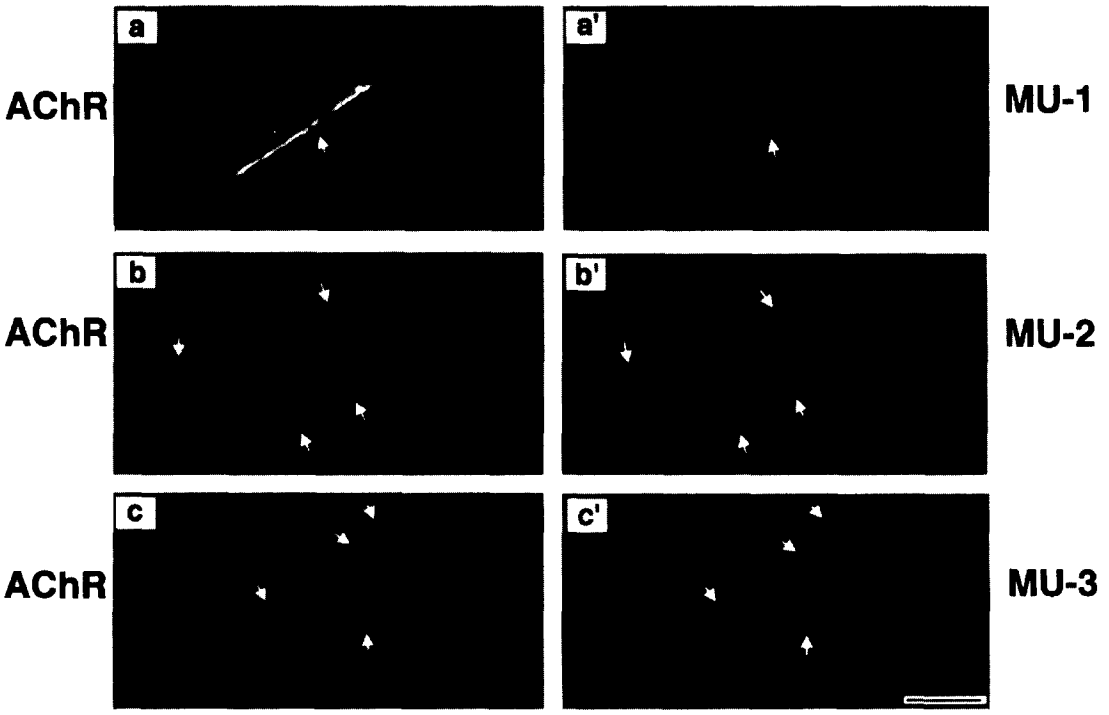

Fig. 5. Association of utrophin mutants with agrin-induced AChR clusters. Panels on the left (a–c) are staining for receptors. Panels on the right are staining for: (a′) MU-1, (b′) MU-2, and (c′) MU-3. An anti-Flag antibody was used. Bar: 20 μm.

rod domain; MU-2 contained most of the central rod domain and C-terminal domain(s); MU-3 contained only C-terminal domain(s), which includes the WW/WWP domain [4], a calcium binding EF-hand domain, a cysteine-rich domain, and the syntrophin binding domain. Expression vectors containing these mutant cDNAs driven by the CMV promoter were transiently transfected into Sol 8 cells. Protein expressions were determined by Western blot analysis and colocalization were examined by immunostaining with the anti-Flag antibody. Both MU-1 and MU-2 were expressed as determined by Western blot analysis (Fig. 4). However, in contrast to MU-1, which was evenly distributed along the plasma membrane (Fig. 5a′), MU-2 was targeted into AChR clusters (Fig. 5b′). A chimera between utrophin and dystrophin with the 3.7 kb N-terminus of the cDNA derived from dystrophin and the rest from utrophin also showed preferential localization with AChR clusters (data not shown). These observations suggested that the N-terminal domain of utrophin was not important for targeting utrophin into AChR clusters in Sol 8 cells. Expression of MU-3 was too low to be detected on Western blots (Fig. 4). However, immunostaining clearly indicated that MU-3 was also targeted into agrin-induced AChR clusters (Fig. 5c′). Interestingly, the majority of myotubes only showed low levels of MU-3 expression. The small number of myotubes with mild to high level expression of MU-3 were morphological altered and were characterized by thin, short, and irregular shapes (data not shown), a possible result of a dominant negative effect.

The above data suggest that domain(s) responsible for targeting utrophin into AChR clusters reside within the C-terminus. N-terminal and central rod domains may play roles in the stability of the utrophin protein and may contribute to the localization of the protein, but are apparently not necessary for targeting utrophin into AChR clusters.

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