Contribution of the different modules in the utrophin carboxy-terminal region to the formation and regulation of the DAP complex

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Abstract The carboxy-terminal region of utrophin, like the homologous proteins dystrophin, Drp2 and dystrobrevins, contains structural domains frequently involved in protein–protein interaction. These domains (WW, EF hands, ZZ and H1–H2) mediate recognition and binding to a multicomponent complex of proteins, also known as dystrophin-associated proteins (DAPs) for their association with dystrophin, the product of the gene, mutated in Duchenne muscular dystrophy. We have exploited phage display and in vitro binding assays to study the recognition specificity of the different domains of the utrophin carboxy-terminus. We found that none of the carboxy-terminal domains of utrophin, when isolated from its structural context, selects specific ligand peptides from a phage-displayed peptide library. By contrast, panning with an extended region containing the WW, EF hands, and ZZ domain defines the consensus binding motif, PPxY which is also found in β-dystroglycan, a component of the DAP complex that interacts with utrophin in several tissues. WW-mediated binding to PPxY peptides and to β-dystroglycan requires the presence of the EF hands and ZZ domain. When the ZZ domain is either deleted or engaged in binding to calmodulin, the utrophin β-dystroglycan complex cannot be formed. These findings suggest a potential regulatory mechanism by means of which the attachment of utrophin to the DAP complex can be modulated by the Ca^{2+}-dependent binding of calmodulin. The remaining two motifs found in the carboxy-terminus (H1–H2) mediate the formation of utrophin–dystrobrevin hybrids but do not select ligands in a repertoire of random nonapeptides.

Key words: Utrophin; Calmodulin; Phage display; Modular domain; Dystrophin-associated protein; DRP2

1. Introduction

Utrophin is a ubiquitous protein that is homologous to dystrophin, the protein associated with Duchenne muscular dystrophy (DMD) [1]. It has been proposed that utrophin and dystrophin have a role in connecting cytoskeletal actin to a multicomponent protein complex known as dystrophin-associated protein (DAP) complex, which is localized at the cell membrane and interacts with the extracellular matrix [1,2]. The carboxy-terminal region of dystrophin mediates the connection with the DAP complex through a series of modular binding domains: a WW domain [3], a region containing EF hands motifs [4], a cysteine-rich domain, containing a putative zinc finger (ZZ) [5] and two regions, H1 and H2, that are predicted to fold into a coiled-coil structure [6]. The modular organization of the carboxy-terminal region and its amino acid sequence are well conserved in utrophin. The protein targets of some of the dystrophin carboxy-terminal domains are already known: the WW domain along with the adjacent EF hands bind to the cytoplasmic tail of β-dystroglycan, a transmembrane component of the DAP complex that interacts with α-dystroglycan on the outer face of the membrane [7–10]. The ZZ domain binds to calmodulin, the major regulator of calcium-dependent kinases [11], while the H1–H2 coiled-coil region is responsible for heterodimerization with the corresponding helices of dystrobrevin [12].

Utrophin is found associated with a different subtype of the DAP complex, including some of the DAPs and synapse-associated proteins such as rapsyn and agrin [13]. The distinct tissue distribution and cell localization suggest that dystrophin and utrophin, although similar, may play different roles and possibly display a different protein binding specificity. Nevertheless, the finding that utrophin can replace dystrophin at the sarcolemma of developing and regenerating muscles [14,15] or in dystrophin-deficient *mdx* mice [16] has emphasized the partially overlapping function of the two proteins and has increased the interest in possible therapeutic use of utrophin for DMD [16,17].

To address these points, we have analyzed the binding potential of the different domains of the carboxy-terminus of utrophin using peptide repertoires displayed on phages and in vitro binding assays with β-dystroglycan, calmodulin and dystrobrevin.

2. Materials and methods

2.1. Constructs

We have used the tools provided by the SMART program to identify the structural domains (and their boundaries) in the carboxy-terminus of utrophin [18]. Different fragments of human utrophin cDNA were amplified by the polymerase chain reaction (PCR) from a fetal brain cDNA library (kindly provided by TIGEM), using specific oligonucleotides, inserting *Bam*HI and *Eco*RI restriction sites. Whenever possible, we took advantage of published expression data of equivalent domains. For the WW domain, for instance, Macias et al. [19] have shown that the minimal WW domain of YAP65, as determined by multiple alignment, cannot fold properly unless it is extended by a heptapeptide at the amino-terminus and a decapeptide

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at the carboxy-terminus. Utrophin fusion constructs that contain isolated domains include: 10 and 15 residues at the N- and C-termini of the WW domain; 20 and 15 residues at the N- and C-termini of the ZZ domain; 60 and 8 residues at the N- and C-termini of the H1–H2 domain.

Amplified fragments were cloned into *Bam*HI and *Eco*RI sites of pGEX2TK vector (Amersham Pharmacia Biotech). cDNA fragments corresponding to the H1–H2 region of dystrophin (aa 3464–3605), DRP2 (aa 745–880) and dystrobrevin (aa 372–505) were subcloned into *Sal*I–*Bam*HI sites of pYEX vector, a derivative of pGEX2TK with a different polylinker. Construct pGEX2TK-DGL, producing the cytoplasmic moiety of human β-dystroglycan fused to glutathione *S*-transferase (GST; aa 774–895), was kindly provided by X. Espanel. Construct His-DGL, directing the synthesis of the corresponding region as a histidine-tagged protein, was obtained by PCR amplification and subcloning of the same β-dystroglycan fragment into *Sac*I/*Sal*I sites of the pQE30 vector (Qiagen). All the constructs were transformed into the protease-defective strain BL21 (DE3). Constructs including either WW, EF hands or ZZ domains were very susceptible to endogenous proteases especially in the linker following the GST protein. This protease susceptibility was already described for the corresponding dystrophin fusion constructs [7,8,20,21]. GST fusion proteins were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma-Aldrich) for only 2 h at 37°C to reduce protein degradation. After cell sonication, a protease inhibitor cocktail (Boehringer Mannheim) was added and the lysates cleared by centrifugation. Supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) following the supplier's protocols.

2.2. Phage display methodology

Library construction and selections were performed as previously described [22]. In the micropanning assay, phages were subjected to a panning cycle: 10^6 transducing particles of each clone were incubated with 5 μg of GST fusion proteins bound to glutathione-agarose for 1 h at 4°C, in PBS, 0.5% Tween 20. After extensive washing, the bound phages were eluted and titered.

2.3. In vitro affinity binding assays

Purified GST–DGL was in vitro labelled with [γ-^{32}P]ATP using bovine heart protein kinase catalytic subunit (P2645, Sigma-Aldrich) and subsequently treated with thrombin protease to release the GST portion (Sigma-Aldrich), according to the manufacturer's instructions. Inspection of the dystroglycan sequence included in our constructs did not reveal residues that are likely to be fully phosphorylated by this kinase [23]. Approximately 10 μg of GST fusion proteins, bound to agarose, were incubated with ^{32}P-DGL (1 μg/ml) in 100 mM NaCl, 160 mM NaH_2PO_4, 350 mM Na_2HPO_4 (PBS), 500 μM phenylmethylsulfonyl fluoride, for 12 h at 4°C. After 5 washes with PBS, samples were analyzed by 5–10% SDS–PAGE and radioactivity estimated using a phosphor-imager (STORM, Molecular Dynamics).

Binding to calmodulin agarose (Sigma-Aldrich) was assayed by incubation of 1 ml of TTBS bacterial extracts (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100), expressing GST–ZZ and GST proteins, with 10 μl of resin suspension (approximately 10 μg of calmodulin), in the presence of 0.2 mM $CaCl_2$ or 50 mM EGTA, for 1 h at 4°C. Proteins retained by the resin were analyzed by SDS–PAGE stained with Coomassie brilliant blue. Competition analysis of β-dystroglycan and calmodulin binding was performed by incubating 1 ml of bacterial extracts containing GST–WWEFZZ, His–DGL or both, with either calmodulin-agarose or glutathione-agarose, for 1 h at 4°C, in the presence of 0.2 mM $CaCl_2$. Samples were separated on 5–10% SDS–PAGE and Western blotting was performed as previously described [7].

2.4. Ligand dot overlay assay

Purified GST–H1H2 fusion proteins (1 and 5 μg) were immobilized onto nitrocellulose (Schleicher and Schuell). After 30 min incubation with blocking solution (PBS, 3% bovine serum albumin, 0.05% Tween 20) filters were incubated with ^{32}P-labelled GST–H1H2–Utr, and GST–H1H2–Dybr (500 ng/ml) in PBS, for 12 h at 4°C. After two washes with PBS, filters were dried and exposed overnight to a radiographic film. The same filters were probed with an antibody against GST to monitor the amount of proteins loaded (data not shown).

3. Results

3.1. Construction of plasmids encoding utrophin GST fusion proteins

In order to study the peptide binding specificity of the structural domains at the carboxy-terminus of utrophin, we engineered a collection of plasmids directing the synthesis of different utrophin fragments as carboxy-terminal fusions to GST (Fig. 1a). Domain borders were determined on the basis of sequence alignment using the SMART program [18] and published data about surrounding sequences, necessary for proper folding of domains [5,6,19].

In agreement with expression experiments of fusion proteins containing different C-terminal fragments of dystrophin [7,8,20,21] we observed more than one GST fusion protein in each expression experiment indicating degradation during expression/purification (Fig. 2). However, at least 50% of the product migrates consistently with the predicted MW of the full-length product and the most protease-susceptible site is the peptide linker following the GST protein. The presence of sufficient amounts of soluble intact fusion proteins to perform binding analysis was evaluated by immunoblotting with anti-GST (Figs. 3b and 4a).

3.2. Phage display analysis

The purified fusion proteins, bound to glutathione-agarose beads, were used to screen multivalent phage peptide libraries, as previously described [22–24]. None of the utrophin constructs containing only isolated domains selected specific peptides from the phage display library. This is particularly surprising for the WW domain since all the WW domains identified and tested up to now, with the exception of dystrophin [7], are functionally independent and able to specifically bind to proline-rich peptide ligands even when isolated from their protein [25–28]. Macias et al. [19] have observed that the

Table 1
Peptides selected by WW-EF-ZZ fusion protein

	L	P	P	P	S	Y	W	S	Q
	D	P	P	P	S	Y	H	A	S	S
	A	P	P	P	S	Y	H	S	S	V
W	F	N	P	P	P	Y	P	G		
F	Y	W	P	P	P	Y	T	A		
S	M	L	P	P	P	Y	P	L	P	
	F	L	P	P	A	Y	R	K	E	
	S	P	P	A	Y	A	S	S	R	
	Y	R	P	P	A	Y	A	N	A	
	R	R	P	P	A	Y	P	G	A	
	R	P	P	P	P	Y	H	P	E	
N	L	R	P	P	A	Y	L	Q		
	R	A	P	P	T	Y	E	R	S	
I	R	S	P	P	P	Y	E	P		
		R	P	P	P	Y	A	R	A	P
	M	R	P	P	P	Y	V	A	P	
	R	R	P	P	A	Y	E	G	W	H	N	V
H	R	P	E	P	P	P	Y	G	N	H	G	H
	V	H	P	P	A	Y	S	Y	Y	G	H	E
	R	R	**P**	**P**	A/P	**Y**						

Sequence alignment of phage-displayed peptides and derived consensus sequence.

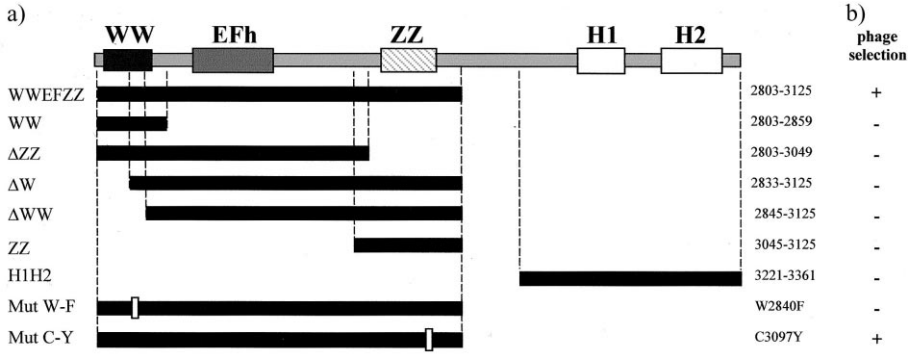

Fig. 1. a: Schematic diagram of the domain organization of utrophin carboxy-terminal region. Names of the GST fusion proteins containing the corresponding utrophin peptides are on the left, amino acids at the extremities of each fragment are indicated on the right. b: Enrichment of phages displaying specific peptide ligands after three panning cycles with the GST fusion proteins linked to glutathione-agarose.

minimal WW domain sequence, as defined by multiple sequence alignment, must be extended at both the amino- and carboxy-side in order to obtain proper folding of the YAP 65-WW domain. We took advantage of this observation in the design of the utrophin WW and ZZ fusion constructs. Notwithstanding this, neither isolated domain selects specific ligands from the nonapeptide repertoire. In contrast, a larger construct (WW-EF-ZZ), which includes the region from aa 2803 to 3125, containing the WW, EF-hands and ZZ domains, selected a family of peptides, which define the consensus motif PPxY (Table 1). At position 3 indicated with an x (any residue), we observe a preference for proline or alanine. Some of the selected peptides also display an arginine at positions −1 or −2 preceding the PPxY motif. Indeed, the sequence RSPPPY represents one of the two PPxY motifs located at the carboxy-terminal end of the cytoplasmic region of β-dystroglycan. The motif PPxY has already been identified as the preferred ligand for group I WW domains [3,27]. Recently, it has been shown that also the dystrophin WW domain requires adjacent regions to bind to the target, β-dystroglycan, through the same RSPPPY core sequence [7].

We found that deletions of either the WW domain (ΔW, ΔWW) or the ZZ domain (ΔZZ) abolishes phage binding (Fig. 1b). To analyze the direct involvement of these domains in the binding to the PPxY motif we changed, by site-directed mutagenesis, specific residues in crucial positions of either the WW or the ZZ domain. To affect the WW binding cleft, we changed the second conserved tryptophan residue of the WW domain (W2840) into phenylalanine. It has already been shown that WW domains of different proteins when mutated in this residue lose their ability to bind the PPxY peptide targets [19,25,26]. The structure of the ZZ domain is not known and it is therefore impossible to rationally plan a mutation that affects its function(s). We chose to substitute the fourth conserved cysteine residue of the ZZ domain (C3097) with tyrosine because this residue is predicted to participate in the formation of the putative zinc finger(s). Furthermore, its functional importance has been suggested by the identification of a substitution in a corresponding position (C3340Y) in the dystrophin gene of a DMD patient [29].

Single point mutants were generated in the construct WW-EF-ZZ and tested by micropanning on phages displaying the I**RSPPPY**EP peptide. We chose this peptide, because it contains six (in bold) out of nine residues identical to the carboxy-terminal sequence of β-dystroglycan. Our results show that phages carrying IRSPPPYEP peptides were efficiently

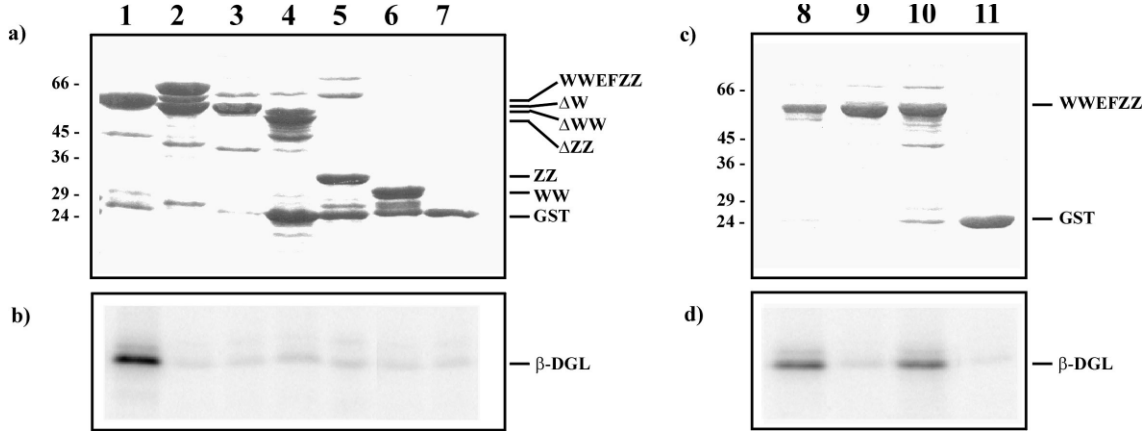

Fig. 2. ^{32}P-labelled β-dystroglycan was incubated with GST fusion proteins: WWEFZZ (1 and 8); ΔW (2); ΔWW (3); ΔZZ (4); ZZ (5); WW (6); mut W-F (9); mut C-Y (10) and GST (7 and 11) as negative control. Retention was detected by SDS-PAGE and Coomassie brilliant blue staining (panels a and c) and autoradiography (panels b and d). Molecular weight markers are indicated on the left while relative positions of the GST-fused proteins and of the β-dystroglycan fragment (DGL) are indicated on the right. An extra band of bacterial origin, which does not stain with anti-GST antibodies (data not shown), copurifies with the ΔW fusion protein (lane 2).

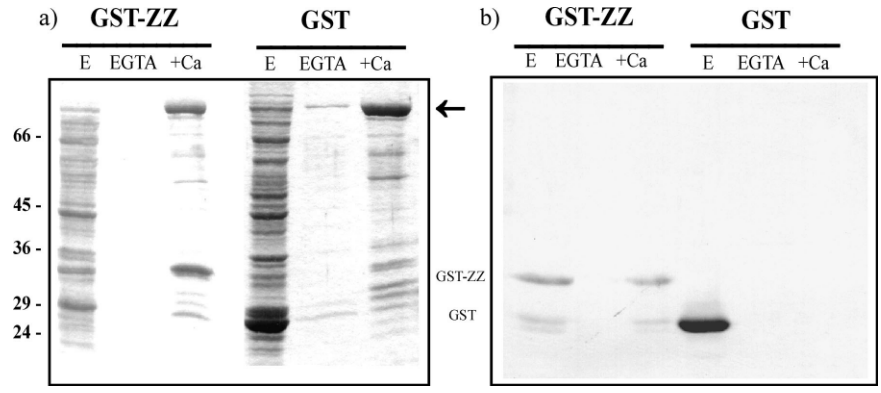

Fig. 3. Binding of utrophin ZZ domain to calmodulin. Bacterial extracts (E) containing GST–ZZ or GST proteins were incubated with CaM-agarose in the presence of EGTA or calcium. Proteins retained on the resin were analyzed by SDS–PAGE stained with Coomassie brilliant blue (a). The arrow points to a high MW band corresponding to a bacterial protein that binds to calmodulin. A gel similar to the one in (a) was transferred to a membrane and probed with an anti-GST antibody (b).

retained by the ZZ mutant protein but not by the mutant affecting the WW domain. M13 wild-type phage was used as negative control (Table 2).

3.3. Binding to the cytoplasmic region of β-dystroglycan

Next, we asked whether the observed requirement for WW and ZZ modules for binding to PPxY peptides could be mirrored by an analogous behavior in binding to the entire cytoplasmic region of β-dystroglycan. We have analyzed this interaction in vitro, using the purified cytoplasmic region of human β-dystroglycan labelled with ^{32}P. The amount of labelled β-dystroglycan retained by the utrophin fragments, bound to glutathione agarose, was analyzed by SDS–PAGE and autoradiography. The results shown in Fig. 2 are in agreement with the phage display data. Neither WW nor ZZ binds, as an isolated domain, to β-dystroglycan. The largest construct (WW-EF-ZZ) binds efficiently, while deletions of the WW domain or of the ZZ domain completely abolish the binding. This is in contrast to data shown for analogous dystrophin constructs, missing the ZZ domain (WW-CRΔZZ), that displayed a residual binding to β-dystroglycan [7].

Moreover, the W-F substitution in the WW domain significantly affects the interaction, while the C-Y mutation in the ZZ domain does not. These results indicate that, as in the homologous protein dystrophin, alteration of the WW domain of utrophin impairs the interaction with the PPPY peptide, present in the β-dystroglycan cytoplasmic tail.

3.4. Binding to calmodulin

Two low-affinity calmodulin binding sites have been identified in the amino-terminal regions of utrophin and dystrophin [30,31]. A third site, with higher affinity, has been described at the carboxy-terminus of mouse dystrophin, in the cysteine-rich region between residues 3293 and 3349 [11]. By analogy, the corresponding region of utrophin has been suggested to have the same binding potential [32]. We have tested this interaction in vitro, by binding to calmodulin-agarose. Utrophin GST–ZZ (aa 3045–3125) specifically binds to calmodulin in a calcium-dependent manner while recombinant GST is not retained (Fig. 3). Thus the ZZ region of utrophin is necessary for binding both to calmodulin and to β-dystroglycan. Next we asked whether the GST–WWEFZZ protein could bind β-dystroglycan and calmodulin simultaneously. The results of the experiment illustrated in Fig. 4 demonstrate that the two ligands are mutually exclusive and that either calmodulin cannot bind to utrophin when already engaged with dystroglycan, or its binding displaces dystroglycan.

3.5. Binding to the H1–H2 region of dystrobrevin

The H1–H2 region is predicted to fold into two tandem α-helical polypeptides and to mediate protein dimerization via formation of coiled-coils [6]. It has been reported that the

Table 2
Micropanning analysis of phages displaying a dystroglycan-like peptide

Target protein	Phage IRSPPPYEP	Phage M13
GST	10^5	10^5
GST–WWEFZZ	10	10^5
GST–WW$^{(W-F)}$EFZZ	5×10^2	10^5
GST–WWEFZZ$^{(C-Y)}$	10	10^5

Numbers indicate the phage input/output rate after a panning cycle. M13 wild-type phage is the negative control.

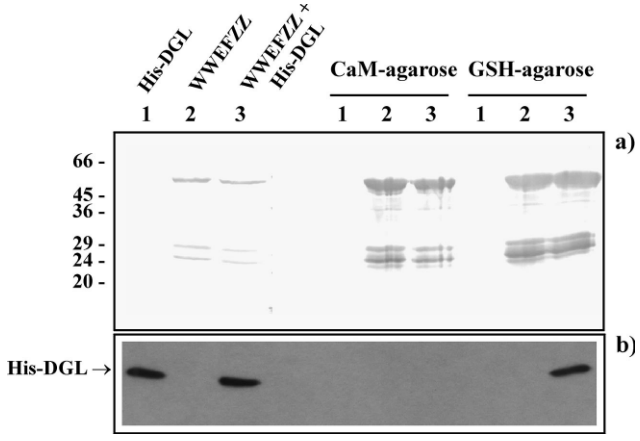

Fig. 4. Competition of utrophin/β-dystroglycan binding by calmodulin. Bacterial extracts containing the cytoplasmic region of β-dystroglycan fused to a poly-histidine tail (1) and the GST–WWEFZZ utrophin fusion protein (2) were mixed (3) and the utrophin fusion protein was purified by affinity either to calmodulin (CaM) agarose or to glutathione (GSH) agarose. Retained proteins were revealed with anti-GST (a) and anti-DGL (b) respectively.

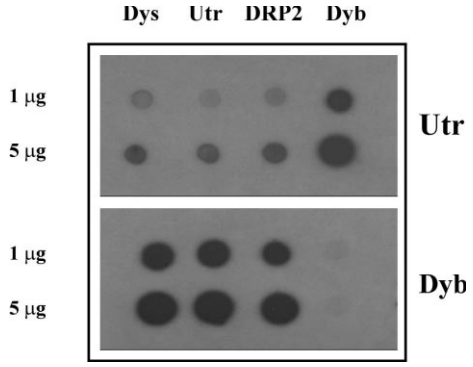

Fig. 5. Dot overlay assay. Fusion proteins containing H1–H2 regions of dystrophin (Dys), utrophin (Utr), dystrophin-related protein 2 (DRP2) and dystrobrevin (Dyb), immobilized on nitrocellulose filters, were tested for binding to ^{32}P-labelled Utr (upper panel) and Dyb (lower panel).

utrophin H1–H2 region, from aa 2753 to 3432, can mediate the formation of heterodimers with α-dystrobrevin-1 in the yeast two-hybrid assay and in a binding assay between in vitro translated proteins [33]. We have confirmed these results by a dot overlay assay (Fig. 5) and we have mapped more precisely the region essential for binding to a short fragment of 141 amino acids (3221–3361).

Utrophin does not heterodimerize with dystrophin or DRP2 H1–H2 domains, while a specific interaction with α-dystrobrevin is confirmed by this technique. The reciprocal experiment, using dystrobrevin as a probe, demonstrates that not only dystrophin and utrophin but also the related protein DRP2 can bind to dystrobrevin.

4. Discussion

The dystroglycan complex, formed by α- and β-dystroglycans, spans the membrane and connects the extracellular matrix to the cytoskeleton. This bridging function is mediated by dystrophin at the sarcolemma and by utrophin at neuromuscular junctions [33,34]. Our analysis revealed that the interaction between utrophin and β-dystroglycan mirrors that of the homologous protein dystrophin and it is mainly mediated by the utrophin WW domain recognizing the PPPY peptide at the carboxy-terminus of the β-dystroglycan. Saito et al. [10] have proposed that a β-dystroglycan peptide of 26 amino acids, located in the membrane-proximal region, increases the binding affinity of the cytoplasmic tail for dystrophin. Rentschler et al. [7] have found that the EF hand-like regions, following the WW domain sequence in the C-terminal region of dystrophin, are necessary for WW binding.

In this report we have shown that the integrity of the utrophin WW-EF-ZZ region is essential for efficient binding to β-dystroglycan and to small peptides selected from a peptide phage library.

Deletion of the ZZ domain from the utrophin construct completely abolishes the binding; the same deletion in an analogous construct of dystrophin only slightly reduces the binding to β-dystroglycan [7]. This discrepancy could be due to the different assay or to specific differences present in the sequences of the two proteins.

Although all the members of the dystrophin family have apparently similar domains, several residues within these domains differ significantly. These amino acids could represent the main determinants of binding specificity for modular protein domains [28].

Several isoforms of dystrophin and utrophin have been identified. Although they are relatively abundant gene products in some tissues, their respective roles are still unclear. Dp71 is a short isoform of dystrophin, containing only the carboxy-terminal region of the protein; a corresponding transcript for the homologous utrophin isoform, Up71, has recently been described in several human and mouse tissues [35]. We have designed the oligonucleotides to generate the constructs ΔWW and ΔW to produce proteins, respectively starting from the proposed translation initiation site of Up71 (aa 2845) and from the point where Up71 and Dp71 diverge (aa 2833). ΔWW completely misses the WW domain, while ΔW retains only half of it. Both proteins were found to be unable to bind to β-dystroglycan (Fig. 2).

The role of the ZZ domain is still unclear. We have shown that this region of utrophin mediates binding to calmodulin, as already described for dystrophin. However the functional relevance of this interaction is not established. More importantly we have shown that the ZZ domain is required for binding to β-dystroglycan. A direct participation of this domain in the binding to the short PPxY peptides, selected from

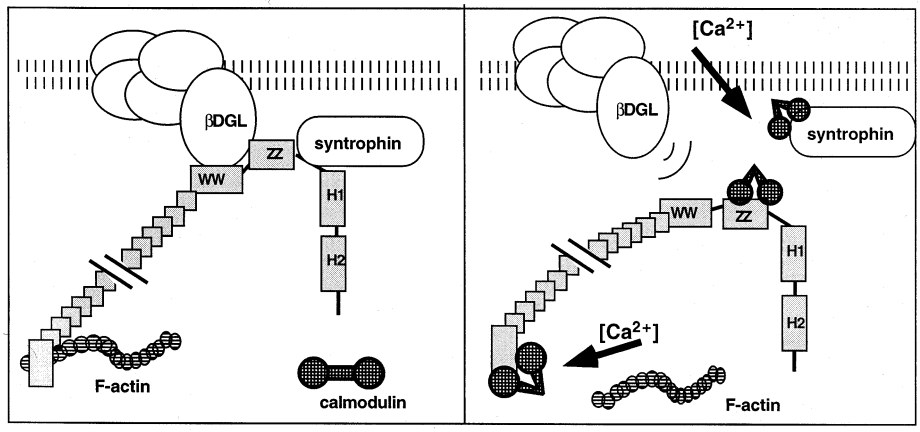

Fig. 6. Schematic representation of regulation of the DAP complex by calmodulin association. The cartoon represents the predicted effects of raising the calcium concentration upon formation of a complex between cytoskeleton, utrophin and the dystroglycan in the presence of calmodulin.

the larger construct WW-EF-ZZ, is unlikely. Computer-aided modeling of the WW domain of dystrophin and utrophin, using the experimentally determined three-dimensional structure of the WW domain of YAP [19] as a scaffold, indicates that the PPPY peptide can adapt to the WW binding cleft without any need to postulate direct contacts with the ZZ domain. Thus we favor an interpretation that advocates a stabilizing or modifying effect of the ZZ domain on the WW structure rather than the formation of direct contacts with the proline-rich target peptide. The important role of the ZZ domain in dystroglycan binding is consistent with our finding that utrophin binding is prevented when the ZZ domain is engaged with calmodulin.

It has been shown that calmodulin inhibits the binding of amino-terminal regions of utrophin (or dystrophin) to F-actin, suggesting calmodulin modulation of the attachment to the cytoskeleton [31]. Moreover, inhibition of in vitro interaction between dystrophin and syntrophin fusion proteins by calmodulin was also reported [32]; in contrast competitive binding of dystroglycan and calmodulin to dystrophin has not been previously tested. The work presented here provides evidence of a further mechanism by means of which also the binding to dystroglycan could be regulated by calmodulin. Taken together all these observations support the model illustrated in Fig. 6 where association of utrophin (and dystrophin) to the DAP complex and cytoskeleton could be modulated by variations in calcium concentration. Whether this mechanism has any functional relevance in vivo remains to be established.

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