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The ZZ Domain of Dystrophin in DMD: Making Sense of Missense Mutations

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Abstract

Duchenne muscular dystrophy (DMD) is associated with the loss of dystrophin, which plays an important role in myofiber integrity via interactions with β -dystroglycan and other members of the transmembrane dystrophin-associated protein complex. The ZZ domain, a cysteine-rich zinc-finger domain near the dystrophin C-terminus, is implicated in forming a stable interaction between dystrophin and β -dystroglycan, but the mechanism of pathogenesis of ZZ missense mutations has remained unclear because not all such mutations have been shown to alter β -dystroglycan binding in previous experimental systems. We engineered three ZZ mutations (p.Cys3313Phe, p.Asp3335His, and p.Cys3340Tyr) into a short construct similar to the Dp71 dystrophin isoform for in vitro and in vivo studies and delineated their effect on protein expression, folding properties, and binding partners. Our results demonstrate two distinct pathogenic mechanisms for ZZ missense mutations. The cysteine mutations result in diminished or absent subsarcolemmal expression because of protein instability, likely due to misfolding. In contrast, the aspartic acid mutation disrupts binding with β -dystroglycan despite an almost normal expression at the membrane, confirming a role for the ZZ domain in β -dystroglycan binding but

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surprisingly demonstrating that such binding is not required for subsarcolemmal localization of dystrophin, even in the absence of actin binding domains.

Keywords

Duchenne muscular dystrophy; dystrophin; missense mutation; ZZ domain

Introduction

The progressive muscular pathology of Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene (MIM #300377; GenBank NM 004006.2) that lead to a partial or total absence of the full-length muscle isoform of dystrophin [Koenig et al., 1988]. This isoform (Dp427) consists of an N-terminal acting binding domain (ABD1); a central rod domain made up of 24 spectrin-like repeats separated by four hinges and containing a second actin-binding domain (ABD2); a cysteine-rich (CR) region; and a C-terminal dimeric coiled-coil region (CT). Dystrophin forms a vital link between the actin cytoskeleton and the extracellular matrix via a transmembrane glycoprotein complex that includes β -dystroglycan (DAG1; MIM #128239). Its deficiency leads to the failure of muscle fiber integrity and results in a degenerative process marked by myofiber necrosis, muscle fibrosis, and failure of regenerative capacity (reviewed elsewhere [Deconinck and Dan, 2007]).

The CR domain (encoded by exons 63–69) includes three distinct domains: the WW domain (named after two conserved tryptophan residues), the EF hand domain, and the ZZ domain. The WW region (aa: 3055–3092) is known to bind a consensus P-P-X-Y motif in its interacting partners, and is the primary site of binding between dystrophin and a proline-rich β -dystroglycan epitope encompassing the C-terminal 15 residues that is necessary and sufficient for direct association with dystrophin [Huang et al., 2000; Jung et al., 1995; Rentschler et al., 1999]. However, the interaction occurs only in the presence of the EF hand, which allows the WW domain to adopt the proper conformation for interaction with β -dystroglycan. The ZZ domain encompasses amino acids 3307–3354 and is characterized by four conserved cysteine residues in two distinct C-X-X-C motifs. This region was so named because of its putative ability to coordinate zinc finger motifs with additional histidine and other potential liganding residues [Ponting et al., 1996]. The ZZ domain is involved in forming a stable interaction between dystrophin and β -dystroglycan [James et al., 2000; Rentschler et al., 1999] and one study suggested that the residues 3326–3332 (YRSLKHF) of ZZ form a crucial part of the contact region between the two proteins [Hnia et al., 2007]. This suggests that there are at least two binding sites in the CR domain of dystrophin that are important for binding to β -dystroglycan, one in the WW domain and another in the ZZ.

The critical importance of the CR domain is demonstrated by both clinical and experimental observations. Becker muscular dystrophy (BMD)—milder than DMD, with a broader range of severity—typically results from *DMD* mutations that allow translation of a partially functional dystrophin protein, in contrast to DMD, where dystrophin is typically absent altogether. Most BMD mutations preserve an open reading frame that allows translation of an internally truncated protein with functional amino- and carboxyl-terminus regions, but a

deletion of the CR domain has never been described in BMD patients, suggesting that it is critical for dystrophin function. This was confirmed by transgenic studies of *mdx* mice, the principle DMD animal model, expressing full-length dystrophin with consecutive deletions, and by injection into *mdx* mice of different micro-dystrophin constructs with or without this domain [Rafael et al., 1996; Scott et al., 2002]. These studies showed that removal of the CT domain was essentially without consequence whereas deletions that removed portions of the CR domain resulted in loss of dystrophin function and severe muscle pathology.

The importance of the CR region is further demonstrated by the distribution of missense mutations in the dystrophinopathies. *DMD* missense mutations are rare, accounting for 1.4% of all dystrophinopathy mutations and only 0.3% of all DMD mutations in one large cohort [Flanigan et al., 2009]. Among the eleven such mutations identified in the ZZ domain (www.dmd.nl), nine of the patients had a severe DMD phenotype. One of these, p.Cys3340Tyr (NM 004006.2:c.10019G>A), involves a mutation in a conserved cysteine residue and was found in a patient with a severe DMD phenotype with reduced expression of both dystrophin (described as 10%–20% of normal) and β -dystroglycan [Lenk et al., 1996]. We previously described another mutation, p.Cys3313Phe (NM 004006.2:c.9938G>T), affecting a highly conserved cysteine in the ZZ domain [Flanigan et al., 2003], associated with similar clinical and histopathologic features, including a muscle biopsy that shows a similar reduced amount of patchy dystrophin staining (Supp. Fig. S1). A third mutation, resulting in an aspartate substitution by histidine, p.Asp3335His (NM 004006.2:c.10003G>C) [Goldberg et al., 1998], was the first case to show a relatively severe DMD phenotype despite dystrophin and β -dystroglycan expression close to a level seen in normal muscle, with an apparently correct localization at the sarcolemma even though the expression detected by Western blot shows a significant decrease of β -dystroglycan signal. This description could suggest that the molecular pathogenesis for this mutation is not due to an interruption of dystrophin/ β -dystroglycan binding. An alternative explanation is that membrane localization of the mutant dystrophin may not be dependent upon β -dystroglycan binding and, despite the apparently normal colocalization these two proteins may not physically interact.

Few studies have investigated how a single altered amino acid leads to the complete dysfunction of the full-length dystrophin protein. In the case of missense mutations in the ABD1 domain, loss of dystrophin function is more likely due to protein instability and misfolding rather than direct loss of actin-binding activity [Henderson et al., 2010; Singh et al., 2010]. These data raise the possibility that the same process occurs with ZZ mutations, and that protein instability rather than altered binding is responsible for muscle pathology. Two groups have investigated the effects of missense mutations within the ZZ domain on its ability to bind β -dystroglycan [Draviam et al., 2006; Ishikawa-Sakurai et al., 2004]. In the case of cysteine mutations, their results differed. One study showed that any cysteine mutation interferes with the ability to properly bind β -dystroglycan [Draviam et al., 2006], whereas the other demonstrated that some but not all ZZ cysteine mutations (along with the non-cysteine p.Asp3335His mutation) preserve binding [Ishikawa-Sakurai et al., 2004].

In order to assess the impact of missense mutations on the ZZ domain, we used plasmid constructs expressing an N-truncated dystrophin similar to the dystrophin isoform Dp71 but

including the full WW domain. We chose this construct in order to analyze the smallest protein known to restore the dystrophin-associated protein complex (DAPC) without any actin binding domains, and to minimize the number of proteins differentially pulled down during co-immunoprecipitation experiments. We investigated in vitro and in vivo the effect these mutations have on binding partners and the stability and folding of the protein.

Materials and Methods

Full experimental details are available in the Supp. Methods, and only briefly described here.

Mutated Dystrophin Constructs

The last 1.9 kb of the mouse dystrophin cDNA, including the complete CR domain and the C-terminus, was cloned in pCMV-script expression vector (Agilent Technologies, Santa Clara, California, USA) and in AAV plasmid (Supp. Fig. S2). This construct is similar in size and structure to Dp71, a nonmuscle dystrophin isoform, which is known to bind the DAPC without alleviating the dystrophic muscle phenotype in transgenic mice [Cox et al., 1994; Greenberg et al., 1994], but includes the full WW domain. Mutations were introduced in the ZZ domain using complementary primers and the QuickChange II Site-directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's instructions, and verified by sequencing.

Coimmunoprecipitation, Western Blotting, Immunostaining, and Mass Spectrometry Analysis

We followed the protocol described in detail in the paper from Johnson et al. (2012). Pulldown was performed using Protein-G agarose beads (Life Technologies, Grand Island, New York, USA) prebound with a monoclonal antibody that recognizes the C-terminal part of dystrophin (aa 3,667–3,671), MANDRA1 [Morris et al., 1998; Nguyen et al., 1992]. Wheat germ agglutinin (WGA) pulldown was performed with streptavidin beads (Life Technologies) pre-enriched with WGA. Eluted protein and native protein lysate (120 μ g) was loaded on a SDS-PAGE gel before transfer to nitrocellulose membrane for Western blotting. ZZ-dystrophin was detected with the MANHINGE4A antibody [Ahmed et al., 1998] or with a rabbit polyclonal antibody against the C-terminal end. β -dystroglycan was detected with the antibody MANDAG2 and Dystrobrevin with a mouse monoclonal pan-antibody. Alpha-1 syntrophin was detected with a rabbit polyclonal antibody. Mass spectrometric analysis (LC-MS/MS) was performed at the Ohio State University Mass Spectrometry and Proteomics Core Facility under conditions described in the Supp. Methods.

Differential Scanning Fluorimetry and Differential Light Scattering

Protein expression, construct cassettes were recombined in the Baculovirus destination vector pDESTTM 8 using Invitrogen's Gateway® technology [Hartley et al., 2000], and transformed into DH10Bac competent *E. coli* yielding bacmid DNA for infection of Sf9 insect cells. Protein was purified using an anti-FLAG M2 agarose column (Sigma A2220) followed by dialysis. Differential scanning fluorimetry (DSF) was performed as described

by Niesen et al [Niesen et al., 2007], in triplicate. differential light scattering was performed on 100ul of protein using a Malvern Instruments Zetasizer μ V.

Tertiary Structure Modeling

The ZZ domain structural prediction was produced by sequence fitting of the ZZ domain residues to SWIM and ZZZ3 ZZ domains (PDB IDs 2DIP, 2FC7), and with the primary sequence of dystrobrevin (PDB IDs 2E5R) due to the similarity in cysteine-residue motifs in these domains.

Results

Cysteine Mutations Result in Diminished Protein Expression

We cloned the portion of the *DMD* gene encoding the C-terminal fragment of the dystrophin protein (encompassing the WW, EF1, EF2, ZZ, and CT domains; Supp. Fig. S2) and introduced three different missense mutations into the ZZ domain by site-directed mutagenesis. In vitro expression of wild-type (ZZ-WT) and mutant constructs was assessed by transfection into 293K cells. Western blot of the supernatant shows amounts of p.Asp3335His close to that of the ZZ-WT construct, whereas amounts of both cysteine mutation constructs are markedly decreased in the supernatant (Fig. 1A). Significant amounts of all the mutant constructs were found in the insoluble (pellet) fraction, confirming expression from the pCMV-delivered transgene, and suggesting that the expressed proteins are not stable.

In order to determine whether the expression pattern is similar in vivo, the same constructs were cloned into an AAV plasmid under control of the MHCK7 promoter to produce an AAV2/8 vector for injection into *mdx* mice. The difference in expression was even more pronounced in vivo as the cysteine mutant proteins were not detectable by Western blot (Fig. 1B), despite the presence of comparable numbers of vector genomes in transfected muscles by quantitative PCR (data not shown). This difference in expression was confirmed by immunostaining (Fig. 2A); p.Asp3335His is properly expressed at the membrane (albeit in diminished amount compared to the WT construct), whereas no expression was detected in the cysteine mutated injected muscles. These results are consistent with the immunohistochemistry described in the published reports of the p.Asp3335His and p.Cys3340Tyr patients [Goldberg et al., 1998; Lenk et al., 1996]. Although there was no significant difference in α -syntrophin and β -dystroglycan protein levels in comparing *mdx* animals treated with ZZ-WT, ZZ-mutant constructs, and nontreated *mdx* animals by Western blot, both proteins were significantly diminished at the plasma membrane of dystrophic muscle fibers (Fig. 2B). The presence of such a high level of β -dystroglycan in *mdx* muscles is consistent with results in a recent paper which discusses the presence of a new dystroglycan complex independent of dystrophin expression [Johnson et al., 2013].

The p.AspP3335His Mutation Affects Binding to β -Dystroglycan

Expression of the ZZ-WT construct following intramuscular injection results in restoration of β -dystroglycan localization at the membrane (Fig. 2B), suggesting that the CR region constructs we used should be sufficient to stabilize β -dystroglycan at the membrane. This

was expected, as it has been demonstrated that Dp71 isoform binds the DAPC, and transgenic mice carrying this isoform on an *mdx* background are able to restore the complex, including β -dystroglycan [Cox et al., 1994; Greenberg et al., 1994]. In contrast, expression of the p.Asp3335His construct shows no significant restoration of β -dystroglycan staining at the sarcolemma. Both constructs show normal membrane localization of α -syntrophin (Fig. 2B), which is known to bind to the downstream CT region to amino acids 3444–3494, corresponding to the exons 73 and 74 [Suzuki et al., 1995].

We next confirmed this absence of β -dystroglycan binding by immunoprecipitation studies. Using the MANDRA1 antibody that recognizes an epitope in exon 77, dystrophin immunoprecipitation was performed on homogenate from injected muscles, and eluted proteins were analyzed by LC–MS/MS. To control for nonspecific protein binding to the dystrophin antibody, each experiment included immunoprecipitation from muscles of untreated *mdx* mice; comparison of mass spectra profiles allowed us to distinguish specific binding partners from contamination. Because of the absence of expression of the cysteine mutated constructs in vivo, mass spectrometry analysis was interpretable only following coimmunoprecipitation of the wild-type and p.Asp3335His mutant constructs. As shown in the Table 1, expression of the WT-ZZ domain results in pull-down of all the members of the dystrophin-associated protein complex (DAPC) known to bind the CR and CT portions of dystrophin. Notably, despite its hydrophobicity we obtained significant peptide coverage of β -dystroglycan in the WT-ZZ sample but not in the p.Asp3335His mutant and *mdx* control. Even with co-IP and MS analysis, the control *mdx* samples do not show traces of DAPC proteins or the shorter dystrophin isoforms expressed by nonmuscle cells. In contrast, immunoprecipitation of the p.Asp3335His-ZZ domain protein results in co-IP of only syntrophins and α -dystrobrevin (which have also been shown to bind to the CT region of dystrophin and syntrophin [Newey et al., 2000; Sadoulet-Puccio et al., 1997]), but not of β -dystroglycan or the sarcoglycans. Similar results are seen with immunofluorescent analysis of injected muscle (Supp. Fig. S3). Specifically, preservation of interactions with syntrophins and α -dystrobrevins suggests that the mutation does not cause a drastic change in configuration that affects all protein binding but is very specific to dystroglycan and the sarcoglycans.

Western blot analysis confirmed these LC–MS/MS profiles for both WT-ZZ and the p.Asp3335His mutant proteins (Fig. 3A and 3B). To support our results, we performed an enrichment of the DAPC from homogenate muscle by WGA binding. This lectin binds to α -dystroglycan and therefore purifies dystrophin only if it interacts with the dystroglycan complex, composed of α - and β -dystroglycan. Dystrophin immunoreactivity is detectable only in WGA pull-downs from muscle injected with the WT-ZZ construct, confirming that the mutant protein is not able to bind β -dystroglycan (Fig. 3C).

The p.Asp3335His Protein is Stably Folded

While the p.Asp3335His mutant appears stable by expression in both 293K culture and in vivo animal model, we wanted to test whether this mutant protein was thermally stable and properly folded in vitro. Thus, we expressed N-terminal FLAG epitope-tagged WT-ZZ and p.Asp3335His in Sf9 insect cells and then purified the recombinant proteins by FLAG

affinity chromatography. The thermal stability of each protein was assessed by DSF. DSF analysis for both WT-ZZ and the p.Asp3335His mutant proteins show a single cooperative transition from folded to unfolded upon thermal denaturation (Fig. 4A), indicative of well-folded proteins in a homogeneous state. The slightly higher melting temperature measured for p.Asp3335His could be interpreted as a small increase in thermal stability for the mutant protein compared to wild type. Alternatively, this shift could be due to p.Asp3335His aggregation, as supported by the larger average hydrodynamic radius for mutant protein versus WT folded protein, measured by dynamic light scattering (Fig. 4B). However, the similar levels of polydispersity for WT-ZZ and p.Asp3335His (Fig. 4B) indicate that the fraction of aggregated p.Asp3335His is small. We conclude that the p.Asp3335His mutation does not dramatically affect the stability or solubility of ZZ.

Discussion

The first missense mutation in a DMD patient was described in 1993 in the dystrophin N-terminal actin-binding domain ABD1 [Prior et al., 1993]. Missense mutations are still quite rare, and approximately half of them are located in ABD1 (www.dmd.nl), where they more frequently lead to a BMD phenotype. Missense mutations located in the CR domain, in contrast, are typically associated with DMD. The CR domain is essential for a functional dystrophin [Bies et al., 1992] and deletions of this region that disrupt the interaction with the DAPC complex (and in particular, β -dystroglycan) lead to a severe phenotype [Rafael et al., 1996; Scott et al., 2002].

Inside the CR domain, two β -dystroglycan binding sites have been described based on in vitro studies [Hnia et al., 2007; Rentschler et al., 1999]. Attempts to assess the impact of ZZ missense mutations on β -dystroglycan binding, performed by different methods, have yielded partially conflicting results [Draviam et al., 2006; Ishikawa-Sakurai et al., 2004]. In one of these [Ishikawa-Sakurai et al., 2004], truncated recombinant wild type and mutant mini-dystrophins (spanning from the WW to the ZZ domain) were expressed in *E. coli* BL21 and studied by in vitro overlay binding assay. The results with wild type constructs demonstrated that the WW domain is required but is not sufficient for β -dystroglycan binding, and that the ZZ domain while not required is essential for full binding activity. In this system, the p.Cys3340Tyr substitution completely abrogated binding activity, whereas the p.Asp3335His mutation exhibited the same binding activity as the wild type control, a finding which is contradictory to our results. A second study [Draviam et al., 2006] used an eGFP tagged version of the mini-dystrophin 3849 [Wang et al., 2000], which contains the N-terminal actin binding domain and a few repeats of the rod domain in addition to the CR region. Versions with cysteine residue mutations were transfected into 293K cells for immunostaining and immunoprecipitation assays. Consistent with our results, they found that none of the ZZ domain cysteine mutant mini-dystrophin proteins were able to bind endogenous β -dystroglycan, in contrast to the earlier results that found that mutations of p.Cys3337 and p.Cys3313 did not abrogate binding [Ishikawa-Sakurai et al., 2004]. Moreover, translocation of the expressed protein to the nucleus was seen with all mutated eGFP constructs but not the wild type one. This led the authors to conclude that mutations in the ZZ domain could generate a nuclear localization signal or change the conformation of the protein that affect the binding partners, leading to a nuclear localization. They also

postulate that in mini-dystrophin constructs including only the CR domain, binding with β -dystroglycan may be more important for the proper localization and the anchoring to the membrane than it is in the case of full-length dystrophin [Cote et al., 2002].

Importantly, neither of these prior studies assessed the effect of the missense mutations on binding partners in vivo, as we have in this study. Each used both differing constructs and different in vitro assays, which may explain their discrepant results regarding p.Cys3337 and p.Cys3313 binding. In vivo, our results show that none of the three reported mutations are able to bind β -dystroglycan (Fig. 3A), but suggest that in the case of cysteine mutations, rather than having a direct impact on binding with DAPC partners, the mutant proteins were unstable and rapidly degraded in vivo. Accordingly, although we could detect expression of each mutant by Western blot analysis and a very small amount of protein was soluble immediately after lysis of the cells, no protein was recovered for either Cys mutant after purification from insect cells pellets. We suspect that the small amount of soluble Cys mutant protein recovered after cell lysis probably aggregates soon after, perhaps even within the chromatography column. Regardless, our failure to recover purified protein for either Cys mutant makes it impossible to assess their misfolding. On the contrary, the p.Asp3335His protein was readily expressed (Supp. Fig. S4). Moreover, after transfection in 293K cells, we saw significant amounts of the mutant proteins in the insoluble fraction, suggesting altered folding leading to protein insolubility. In vivo, we did not detect expression of the cysteine mutant proteins. We attribute the difference between in vivo and in vitro mutant protein expression to overexpression in the artificial setting of transfection, which can saturate or overwhelm the quality control machinery, probably inducing unfolded protein response-mediated remodeling of the endoplasmic reticulum [Claessen et al., 2012; Ron and Walter, 2007]. It could also be explained by a more rapid translation in vitro that allows assessment of changes in secondary structure, whereas biological systems focus on the slower tertiary and quaternary structure formation [Braakman and Bulleid, 2011]. Importantly, although we acknowledge the difference between our Dp71-like protein and the full-length Dp427 isoform, our in vivo finding is consistent with clinical reports of DMD patients harboring these cysteine mutations in whom dystrophin is drastically reduced at the membrane of muscle fibers [Lenk et al., 1996].

We suspect that in the case of cysteine mutations, the rapid degradation may be due to misfolding. As discussed below, our putative model places four of the cysteines in the ZZ domain in a tetrahedral arrangement consistent with these residues functioning as a tetra-cysteine transition-metal binding site. As with disulfide bonds, a tetra-cysteine metal-binding site contributes to protein stability and these are a common stabilization motif used in many proteins (including anything with a zinc finger). Replacing one of the cysteines with anything but a histidine will eliminate metal binding, and we would expect such a substitution to reduce or eliminate stability, resulting in misfolding and (if not repaired by molecular chaperones) in rapid degradation. We note that misfolding rather than loss of binding is analogous to the case of missense mutations in the ABD1 domain, which had originally been hypothesized to alter binding to cytoskeletal actin but instead result in disease by protein instability and degradation [Henderson et al., 2010; Singh et al., 2010].

The mechanism of pathogenesis for p.Asp3335His is apparently different. The DSF data show that despite the slight difference in the conformation between the wild-type and the mutated proteins, they both appear to be equally thermally stable. This change in conformation, highlighted by the DSL, could indicate how a residue not apparently directly implicated in β -dystroglycan binding [Hnia et al., 2007; Huang et al., 2000] could still disrupt this interaction.

We created a possible model based on the interaction of the predicted structure of a relevant ZZ domain, positioned against the experimentally determined structure of the dystrophin WW domain and a cocrystallized β -dystroglycan fragment (Supp. Fig. S5). Due to the size and shape of the WW domain, the limited size of the ZZ domain, and the fact that the ZZ domain structure begins immediately after the crystalized C terminus of the WW domain, it is unlikely that either the p.Cys3313Phe, p.Cys3340Tyr, or the p.Asp3335His mutations are directly involved in modifying binding of β -dystroglycan at its known cocrystallized location on the WW domain. Based upon this model it is also unlikely that the ZZ domain YRSLKHF motif interacts with this same fragment of β -dystroglycan, and if both the cocrystallized β -dystroglycan contact and the YRSLKHF contact are biologically relevant, it is likely that dystrophin makes separate contacts with β -dystroglycan by using both the ZZ and WW domains. Although disruption of the putative zinc-binding tetrad by p.Cys3313Phe or p.Cys3340Tyr does not directly affect the putative β -dystroglycan contact residues in the ZZ domain, it would lead to destabilization of the loop containing YRSLKHF, potentially affecting the binding geometry or accessibility of these residues.

p.Asp3335His may also affect the conformation or stability of the YRSLKHF loop or, due to its proximity, may be involved in an unknown interaction with β -dystroglycan. Interestingly, the p.Asp3335His mutated protein is expressed at a level close to normal and seems to be properly localized at the membrane. Others studies have shown that β -dystroglycan binding is not indispensable for anchoring dystrophin to the membrane [Cote et al., 2002; Helliwell et al., 1992; Hoffman et al., 1991; Recan et al., 1992], but in all these studies the dystrophin possessed the N-terminal and rod domains, which is not the case in our constructs. It is now well described that dystrophin can bind the lipid bilayer via interaction with F-actin through ABD1 and ABD2 [Amann et al., 1998; Rybakova et al., 2006] but not directly through the C-terminal third portion of the protein, although its presence enhances binding activity [Henderson et al., 2012].

Our results with the p.Asp3335His mutated protein are consistent with a previously hypothesized mechanism of membrane anchoring that is independent of dystroglycan and actin but that could be mediated by α -syntrophin [Cote et al., 2002]. Several studies have shown that dystrophin and syntrophin directly interact with each other and different binding sites have been identified [Castello et al., 1996; Kramarcy et al., 1994; Yang et al., 1995]. How α -syntrophin could facilitate the anchoring of dystrophin at the membrane is not well established but it could be via a voltage-gated sodium channel [Gee et al., 1998] and the receptor tyrosine kinase ErbB4 which both interact with the syntrophin PDZ domain [Garcia et al., 2000; Newey et al., 2000]. α -Syntrophin may also anchor dystrophin at the membrane through an interaction with nNOS and caveolin-3 [McNally et al., 1998; Venema et al., 1997], which has a β -dystroglycan binding site and is upregulated in DMD patients

[Brenman et al., 1996]. We detected an upregulation of caveolin-3 in all our *mdx* muscles, but were not able to pull down the protein with either the mutated or wild-type dystrophin (data not shown); this correlates with evidence that caveolin-3 is not an integral component of the DAPC but might interact transiently [Crosbie et al., 1998]. Testing whether the localization of the p.Asp3335His protein at the membrane is mediated via α -syntrophin binding requires expression in *mdx:Snta*^{-/-} double knockout mice, experiments which are currently being pursued.

Our findings highlight the importance of the ZZ domain to normal dystrophin function, and demonstrate at least two different molecular pathogenic pathways for missense mutations that were not previously described in this domain. The observation that cysteine mutations in particular predispose the dystrophin protein to degradation extends recent observations that show that mis-sense mutations in the N-terminal domains result in similar protein instability [Henderson et al., 2010; Singh et al., 2010]. This mechanism contrasts with that of the apparently stably folded p.Asp3335His protein, which localizes to the membrane without binding β -dystroglycan, confirming that localization alone is not sufficient to maintain muscle integrity and emphasizing an important role of β -dystroglycan in membrane protection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Mutant and normal-ZZ proteins expression. **A:** In vitro expression. After transfection with the different pCMV-ZZ constructs, 293 K cells were lysed under denaturing conditions for western blot analysis. Boxed images show the dystrophin expression in the supernatant and in the pellet, and β -dystroglycan was used as a loading control as 293 K expresses this protein. **B:** In vivo expression. Four weeks after injection with wild-type and mutants AAV8-ZZ constructs, TA muscles were removed and digested for Western blot analysis. The membranes were blotted with dystrophin, β -dystroglycan, α 1-syntrophin, and α -actinin (as a loading control) antibodies. PBS refers to PBS-injected *mdx* muscles.

Figure 2.

Immunostaining on normal and injected TA *mdx* muscle sections. **A:** Dystrophin expression on a normal Bl6, *mdx* muscles and *mdx* injected muscles with the four AAV8-ZZ constructs.

B: β -Dystroglycan and alpha-syntrophin staining on Bl6, *mdx* control and *mdx* injected muscles with the wild-type-ZZ (ZZ-WT) and the p.Asp3335His mutated constructs.

Dystrophin expression is properly localized at the membrane for the wild-type and the p.Asp3335His dystrophin constructs but only ZZ-WT restores β -dystroglycan close to the normal. Injected muscles with the cysteine mutated constructs showed no dystrophin staining, except the few revertant fibers. The two secondary antibodies Ila (antimouse) and Iib (antirabbit) show the background expected. Sections are counterstained with DAPI (blue) to show nuclei.

Figure 3.

Coimmunoprecipitation following intramuscular injection. Western blot following coimmunoprecipitation. Four weeks after injection, TA muscles were removed and proteins were extracted for co-IP experiments to pull down WT-ZZ and mutants binding partners. **A:** Wild-type and p.Asp3335His dystrophin are expressed but β -dystroglycan is pulled-down only with the wild-type protein. (Nonspecific bands are seen in all lanes, presumably including the IgG heavy chain.) Consistent with the results of the LC MS/MS analysis (Table 1), binding with (A) α 1-syntrophin and (B) α -dystrobrevin (α -DTNA) is present for both constructs. As expected, none of the cysteine mutated proteins are expressed and able to immunoprecipitate known dystrophin binding partners. **C:** WGA pulldown. Representative immunoblot for β -dystroglycan and dystrophin from digitonin-solubilized skeletal muscle enriched for the DGC using WGA-streptavidin beads.

Figure 4.

Differential scanning fluorometry (**A**) and differential light scattering (**B**) measurements of WT and p.Asp3335His mutants. **A:** The fluorescent dye binds the hydrophobic regions that are exposed when the protein unfolds and emission is measured as temperature increases from 20°C to 90°C. Despite the minor difference in inflection point between ZZ-WT (black) versus p.Asp3335His (gray), the single cooperative transition indicates a correctly folded p.Asp3335His mutant protein in a homogeneous state. **B:** Light scattering from protein samples is measured by the Malvern Instruments Zetasizer μ V. The software reports the hydrodynamic radius of the particle as a Z-average in nm and the polydispersity Index (Pdl) of the solution on a scale of 0 to 1 where 0 is perfectly uniform in size.

Table 1

Relevant Binding Partners Detected by Mass Spectrometry of the p.Asp3335His Mutant

Protein	UniProt ID	Replicate	ZZ-WT			D3335H		
			Score	% Cov	Pep#	Score	% Cov	mdx Score
Dystrophin	P11531	1	2603	10.5	28	1363	4.8	14
		2	2177	9.4	23	1153	6.3	15
		3	1996	8.3	21	1365	4.2	13
Dystroglycan	Q544G5	1	37	11.4	6	-	-	-
		2	35	11.2	6	-	-	-
		3	81	5.9	3	-	-	-
α -Sarcoglycan	Q5SWB2	1	317	13.2	4	-	-	-
		2	167	8.3	3	-	-	-
		3	121	11.6	2	-	-	-
β -Sarcoglycan	P82349	1	30	10.3	2	-	-	-
		2	47	10.3	2	-	-	-
		3	250	11.1	2	-	-	-
δ -Sarcoglycan	P82347	1	266	9	2	-	-	-
		2	201	5.5	1	-	-	-
		3	265	14.1	3	-	-	-
γ -Sarcoglycan	P82348	1	180	11.3	2	-	-	-
		2	240	11.3	2	-	-	-
		3	1190	38.5	16	97	6.7	2
α -Dystrobrevin	E9QIX4	1	520	14.2	8	48	2.1	1
		2	472	17.6	8	228	5.2	3
		3	1007	45.1	15	1129	41	13
α 1-Syntrophin	A2AKD7	1	607	21.3	6	1205	33.4	11
		2	673	23.9	7	1292	44.9	14
		3	-	-	-	-	-	-

This set of experiments was done in three technical triplicates (1,2,3). UniProt ID: UniProt protein identifier; Score: Mascot protein score; % Cov: percent protein sequence coverage; Pep#: Number of unique peptides. Most of the members of the DAPC were present in the ZZ wild-type samples whereas only α 1-syntrophins and α -dystrophin were pulled down with D3335H mutant ZZ-construct. Alpha-actinin was used as a loading control.