Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection

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α-dystroglycan is a highly *O*-glycosylated extracellular matrix receptor that is required for anchoring of the basement membrane to the cell surface and for the entry of Old World arenaviruses into cells. Like-acetylglucosaminyltransferase (LARGE) is a key molecule that binds to the N-terminal domain of α-dystroglycan and attaches ligand-binding moieties to phosphorylated *O*-mannose on α-dystroglycan. Here we show that the LARGE modification required for laminin- and virus-binding occurs on specific Thr residues located at the extreme N terminus of the mucin-like domain of α-dystroglycan. Deletion and mutation analyses demonstrate that the ligand-binding activity of α-dystroglycan is conferred primarily by LARGE modification at Thr-317 and -319, within the highly conserved first 18 amino acids of the mucin-like domain. The importance of these paired residues in laminin-binding and clustering activity on myoblasts and in arenavirus cell entry is confirmed by mutational analysis with full-length dystroglycan. We further demonstrate that a sequence of five amino acids, Thr[317]ProThr[319]ProVal, contains phosphorylated *O*-glycosylation and, when modified by LARGE is sufficient for laminin-binding. Because the N-terminal region adjacent to the paired Thr residues is removed during posttranslational maturation of dystroglycan, our results demonstrate that the ligand-binding activity resides at the extreme N terminus of mature α-dystroglycan and is crucial for α-dystroglycan to coordinate the assembly of extracellular matrix proteins and to bind arenaviruses on the cell surface.

muscular dystrophy | posttranslational modification | phosphorylation of *O*-mannosyl glycan | glycoprotein–ligand interaction | Lassa fever virus

Dystroglycan is a transmembrane protein that links the extracellular matrix (ECM) to the cytoskeleton (1, 2) and is involved in a variety of physiological processes, including maintenance of skeletal muscle membrane integrity and the structure and function of the central nervous system (3). Dystroglycan is encoded by a single gene (dystroglycan 1, *DAG1*) and is cleaved into α- and β-dystroglycan subunits by posttranslational processing (3). α-Dystroglycan is a highly glycosylated, extracellular subunit that binds ECM proteins, including laminin, agrin, neurexin, and perlecan, that possess laminin globular (LG) domains (3, 4). The transmembrane subunit, β-dystroglycan, anchors α-dystroglycan to the plasma membrane and binds dystrophin within the cell. α-Dystroglycan is composed of three domains: an N-terminal domain (DGN), a mucin-like domain, and a C-terminal domain (Fig. 1*A*) (1, 3).

The mucin-like domain of α-dystroglycan contains a large number of Ser and Thr residues, which are potential sites for *O*-linked glycosylation. Early biochemical studies suggested that *O*-linked glycans on α-dystroglycan are required for its ligand-binding activity to the ECM (5). Protein-*O*-mannosyltransferase 1 and 2 and protein-*O*-mannosyl N-acetylglucosaminyltransferase 1 are involved in the synthesis of the *O*-mannosyl glycan on α-dystroglycan (3). Notably, patients with homozygous or compound heterozygous mutations in the encoding genes have dramatically

reduced α-dystroglycan ligand-binding activity and typically develop Walker–Warburg syndrome (WWS) or Muscle-Eye-Brain disease (MEB) (4, 6). A similar loss of dystroglycan function as a consequence of abnormal glycosylation is observed in Fukuyama congenital muscular dystrophy (FCMD), limb-girdle muscular dystrophy type 2I, and congenital muscular dystrophies 1C and 1D (4, 6). Although fukutin, fukutin-related protein, and like-acetylglucosaminyltransferase (*LARGE*) have been identified as the genes responsible for these diseases, the exact roles of their respective gene products have yet to be elucidated.

Dystroglycan also serves as a receptor for Old World arenaviruses, including the highly pathogenic Lassa fever virus (LFV), most isolates of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), and the African arenaviruses Mobala and Mopeia, as well as for New World clade C arenaviruses (7, 8). LFV is the causative agent of severe hemorrhagic fever in humans, a disease with a mortality rate of ~15%. Resulting in more than 300,000 infections and several thousand deaths each year, LFV infection represents a major threat to human health, a severe humanitarian problem, and a potential weapon of biowarfare (9). The natural hosts of LFV and LCMV are rodents, with human infections resulting from exposure to infectious material from rodents or human-to-human transmission. Host-interaction and pathogenesis studies for LCMV have led to the development of novel concepts in immunology and virology that extend to human infections by agents including HIV, hepatitis C virus, influenza virus, other animal viruses, bacteria, and parasites (10). The binding sites of the arenaviruses LFV and LCMV map to the first half of the mucin-like domain of α-dystroglycan and overlap with the ECM ligand-binding domain on α-dystroglycan (11, 12). As is the case for ECM protein binding, the ability of dystroglycan to function as a cellular receptor for arenaviruses depends on its posttranslational glycosylation (12). In addition, dystroglycan has been identified as a component of a cellular receptor complex for *Mycobacterium leprae*, the causative agent of leprosy, on Schwann cells (13).

Fig. 1. Laminin binding depends on the first 24 amino acids of the α-dystroglycan mucin-like domain. (*A*) Dystroglycan protein constructs, represented schematically. α-Dystroglycan is composed of a signal peptide (SP), an N-terminal domain, a mucin-like domain, and a C-terminal domain. (*B*) Laminin-binding epitopes are located within the first 24 amino acids of the mucin-like domain in α-dystroglycan. These constructs were expressed in TSA201 cells, and DGFc proteins were purified from the culture medium and analyzed by Western blotting with antibodies against Fc and the glycosylated form of α-dystroglycan (IIH6) and by LM OL. Apparent molecular masses (kDa) are indicated.

The *Large^myd* mouse, which harbors a spontaneous deletion mutation in the *Large* gene, is defective in α-dystroglycan glycosylation and develops both muscular dystrophy and brain abnormalities (4, 14). Skeletal muscle and brain from *Large^myd* mice and patients harboring mutations in the *LARGE* gene have reduced α-dystroglycan laminin-binding activity (4, 6, 14, 15). These reports indicate that LARGE is involved in the posttranslational pathway for α-dystroglycan glycosylation. During the posttranslational maturation of α-dystroglycan, LARGE binds to the DGN, and disruption of this interaction results in aberrant α-dystroglycan glycosylation (16). In addition, a *DAG1* missense mutation (c. C575→T, T192M) found in a patient with limb-girdle muscular dystrophy and cognitive impairment diminishes this interaction (17). Thus, the molecular interaction between DGN and LARGE is an essential step for the expression of functional α-dystroglycan. Despite the importance of DGN for the posttranslational modification of α-dystroglycan, DGN is cleaved by a furin-like protease and released from the mature form of α-dystroglycan (16).

We recently found that a biosynthetic pathway involving phosphorylation on an *O*-mannosyl glycan is required for maturation of α-dystroglycan to its laminin-binding form and that LARGE is crucial for modifying α-dystroglycan beyond the phosphodiester moiety (18). LARGE is composed of two domains that share homology with glycosyltransferases (14). The conserved DXD (Asp-X-Asp) motifs are involved in the catalytic activities of many glycosyltransferases; mutations in these motifs diminish LARGE-dependent modification of α-dystroglycan (19). In addition, adenovirus-mediated gene transfer of LARGE results in significant enhancement of the laminin-binding activity of α-dystroglycan in fibroblasts or myoblasts from WWS, MEB, and FCMD patients (20). These findings suggest that LARGE plays a fundamental role as a glycosyltransferase attaching laminin-reactive moieties to phosphorylated *O*-mannosyl glycans on α-dystroglycan and that LARGE is a suitable candidate molecule for the development of therapeutic strategies applicable to several forms of glycosylation-deficient muscular dystrophies.

Specific Ser and Thr residues that are essential for α-dystroglycan's ligand-binding activity are still unknown. In this study, we localized the region essential for the dystroglycan glycosylation event that is required for its function as a laminin and arenavirus receptor. Using biochemical analyses, we demonstrated

that the ability of α-dystroglycan to bind laminin and arenaviruses depends on Thr residues located at the extreme N terminus of its mucin-like domain. Moreover, within this region, we show that Thr residues in close proximity to one another (Thr-317 and -319) are crucial for the expression of functional dystroglycan. These Thr residues lie just C-terminal to the LARGE-recognition domain. We demonstrated the biological significance of the Thr residues in this region by testing ligand binding, laminin clustering at the cell surface, and arenavirus entry into cells. Thus, we propose that these highly conserved Thr residues are the key determinants of the α-dystroglycan modification that enables this protein to bind ligand (i.e., for its functional glycosylation).

Results

Dissection of the LARGE-Dependent Modification Site(s) in α-Dystroglycan. To localize the functionally important modification sites on α-dystroglycan, we generated deletions or point mutations in the first half of the mucin-like domain, where LARGE-dependent modification of α-dystroglycan takes place (16). Because the DGN is necessary for the interaction with LARGE, all constructs were designed to include it. All mutants were constructed with a C-terminal fusion tag encoding the heavy-chain constant (Fc) moiety of human IgG1 (DGFc) to enable purification of recombinant protein (Fig. 1*A*). When these DGFc proteins were expressed in TSA201 cells (of human embryonic kidney origin), the mature forms were secreted into the culture medium (16). These proteins were purified from the medium using protein A beads and then were analyzed by Western blotting using (*i*) the monoclonal antibody IIH6, which recognizes the glycosylated form of α-dystroglycan and inhibits its ligand-binding activity, and (*ii*) an anti-Fc antibody, which binds the Fc moiety on the α-dystroglycan mutants independent of posttranslational modifications and thus provides an index of protein loading (16). In addition, the ligand-binding activity of α-dystroglycan was examined by a laminin overlay assay (LM OL). To localize the minimal region of α-dystroglycan required for its glycosylation, we first generated DGFc deletion mutants, DGFc340 and DGFc361, with deletions in the mucin-like domains that end at Pro-340 and Pro-361, respectively (Fig. 1*A*). Both DGFc340 and DGFc361 were found to react with IIH6 and to bind laminin, whereas a deletion mutation of DGFc340 (DGFc340 Δ316–322) clearly reduced the ligand-binding activity as well as IIH6 reactivity (Fig. 1*B*). Consistent with a previous report (16), both IIH6 and laminin bound primarily to moieties of higher molecular weight rather than to the major bands detected with anti-Fc antibodies, indicating that only a small subpopulation of the expressed DGFc protein enters the pathway for functional maturation. For epitope mapping, our results indicate that one of the minimal ligand-binding epitopes is located between residues Thr-317 and Pro-340.

The region between Thr-317 and Pro-340 harbors six potential *O*-glycosylation sites, including one Ser and five Thr residues. To determine which of these sites are required for LARGE-dependent glycosylation, we generated finer deletions and point mutations in DGFc340. These mutants were expressed with LARGE because LARGE coexpression enhances functional glycosylation and thus results in a higher sensitivity of detection (16). However, IIH6 did not react with either DGFc321 or DGFc340 Δ316–322, even when LARGE was overexpressed (Fig. S1*A*). In the case of DGFc326, some reactivity was detectable, but the intensity was much lower than that for DGFc340. However, a point mutant of DGFc326 (DGFc326 T322A) was glycosylated at a level similar to that of DGFc326 (Fig. S1*A*). These results suggest that Thr-322 is not one of the glycosylation sites in DGFc326 and that not only glycosylation sites but also surrounding amino acid residues influence functional glycosylation. We thus generated a shorter deletion construct (DGFc334) that lacks Pro-335 through Pro-340, thereby removing Ser-336 from DGFc340. DGFc334 was glycosylated at a level similar to DGFc340 (Fig. S1*B*), indicating that Ser-336 does not contribute greatly to LARGE-dependent modification of DGFc340. Together, these data demonstrate that the first 18

residues (Thr-317 to Pro-334) of the mucin-like domain are necessary and sufficient for functional glycosylation of α-dystroglycan. They also suggest that four specific Thr residues (Thr-317, -319, -328, and -329) may be involved in this process.

Residues Proximal to the LARGE-Binding Domain Are Crucial for Functional Glycosylation of α-Dystroglycan. We generated Ala-replacement point mutations of each of the Thr residues (Thr-317, -319, -328, -329, and -322) in DGFc334 (Fig. S1B). Surprisingly, none of the single point mutations resulted in a dramatic change in LARGE-dependent glycosylation, although T317A and T319A each showed a slight reduction of both IIH6 reactivity and laminin-binding activity (Fig. S1B). This result led us to hypothesize that the replacement of a single Thr with Ala may be compensated by a closely localized Thr residue, with Thr-317/319 serving as one compensatory unit and Thr-328/329 as another. We tested this hypothesis by generating two sets of double mutants with substitutions in these residue pairs. The T317A/T319A mutations impaired the laminin-binding activity as well as the IIH6 reactivity of DGFc334. In T328A/T329A the level of laminin binding was reduced slightly relative to the levels for other double-mutant pairs (Fig. S1C).

To test further if the Thr pairs described above (Thr-317/319 and Thr-328/329) contribute to functional glycosylation on the first half of the α-dystroglycan mucin-like domain, we introduced single or double (Thr-317/319 and Thr-328/329) mutations into DGFc3. The single mutants T317A and T319A showed a slight reduction of IIH6 reactivity and laminin binding compared with WT DGFc3, and this effect was more pronounced in the double mutant T317A/T319A as well as in a deletion mutant DGFc3 Δ316–322. Ligand-binding activity, however, was detectable in the other double mutant, T328A/T329A (Fig. S2). These results indicate that the Thr-317/319 pair plays a crucial role in α-dystroglycan's function as a laminin receptor, whereas, in the context of the entire mucin-like domain, Thr-328/329 contributes only minimally to the ligand-binding activity.

Thr-317 and -319 Residues Serve as Direct Targets of LARGE-Dependent Modification. The region containing Thr-317/319 is highly conserved among vertebrates and is located just beyond the DGN (Fig. S3), the entity that is recognized by LARGE. Moreover, this interaction between the DGN and LARGE is necessary for functional glycosylation of α-dystroglycan. Notably, DGN is cleaved by a furin-like protease during the posttranslational maturation of dystroglycan (16). We hypothesized that Thr-317 and -319 residues near the LARGE-recognition domain (i.e., the DGN) are required for and/or able to promote functional glycosylation of the downstream Thr-rich sequence. To test this hypothesis, we inserted a sequence of five amino acids consisting of Thr³¹⁷ProThr³¹⁹ProVal (TPTPV) between the DGN and a Thr-rich sequence derived from the part of the mucin-like domain (409–485 amino acids) that normally is dispensable for expression of functional dystroglycan (Fig. 2A). When the fragment 409–485 was fused to the DGN in the absence of the TPTPV sequence (DGFcF), the glycosylation moiety required for the protein's laminin-binding function was not detected. The TPTPV (DGFc321) sequence itself did not lead to any glycosylation of the protein, even when fused to the DGN. On the other hand, insertion of the TPTPV in front of the fragment 409–485 (DGFcF+) effectively restored functional glycosylation (Fig. 2A). In addition, substitutions of the paired Thr residues in the TPTPV sequence abrogated laminin binding as well as IIH6 reactivity (Fig. S4), indicating that the Thr residues are crucial for functional modification on the DGFcF+ protein.

To examine whether the LARGE-dependent modification takes place directly on the TPTPV sequence, we generated the DGFc construct DGFcF+ IEGR by introducing a Factor Xa cleavage site (Ile-Glu-Gly-Arg) between the TPTPV sequence and the fragment 409–485 in DGFcF+ (Fig. 2B, Upper). We confirmed that the insertion of Factor Xa recognition site did not affect laminin-binding glycosylation of DGFcF+ (Fig. 2B). The DGFc mutant DGFcF+ IEGR was incubated with Factor Xa to
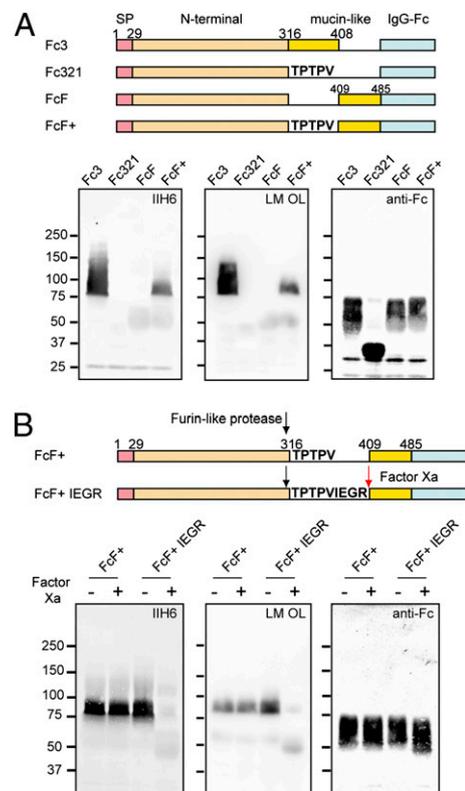
Fig. 2. The TPTPV sequence is a site of direct modification in vitro. (A) The TPTPV sequence is crucial for attachment of laminin-binding glycans to α-dystroglycan. The TPTPV sequence was inserted between the N-terminal domain and residues 409–485 of DGFcF. Schematic representations of the mutant proteins are shown above the images of the blots. (B) The inserted TPTPV sequence in DGFcF+ bears laminin-reactive glycans. A Factor Xa recognition sequence (Ile-Glu-Gly-Arg) was inserted between the TPTPV sequence and residues 409–485 of DGFcF+. After Factor Xa cleavage, Fc proteins retained on protein A beads were analyzed by Western blotting with IIH6 and anti-Fc antibodies and by LM OL. Schematic representations of the mutant protein are shown above the images of the blots.

release the TPTPV sequence from the Fc moiety, and the material retained on Protein A-agarose beads was analyzed by Western blotting with IIH6 and by LM OL. The biochemical analysis showed that the levels of IIH6 reactivity and laminin binding to the Fc moiety derived from DGFcF+ IEGR were considerably decreased by digestion with Factor Xa (Fig. 2B). This result pinpoints the TPTPV sequence as a direct site for LARGE-dependent, functional modification of the DGFcF+ protein.

Phosphorylation of O-Mannosyl Glycans Takes Place in the TPTPV Sequence of the Mucin-Like Domain. Recently, we identified a phosphorylated O-mannosyl glycan on α-dystroglycan that is required for laminin-reactive modification by LARGE (18). Analysis of α-dystroglycan from rabbit skeletal muscle using inorganic phosphate showed that native α-dystroglycan contains ~5 mol of phosphate per mole of protein (18). To examine if the TPTPV sequence is phosphorylated specifically, we performed an in vitro ³²P-orthophosphate labeling assay on a series of DGFc mutants (Fig. 3A), labeling HEK293 cells expressing DGFc proteins with ³²P-orthophosphate. Incorporation of ³²P took place on DGFc mutants containing the N-terminal end of the mucin-like domain (317–340), but not on Fc0, Fc2, FcC (α-dystroglycan C terminus), or DGFcF, each of which lacks the first half of the mucin-like domain (Fig. 3B, and Fig. S5). The intensity of the ³²P signal was comparable in DGFc6 (α-dystroglycan lacking DGN) and DGFc5, suggesting that the DGN is dispensable for the phosphorylation of O-mannosyl glycans
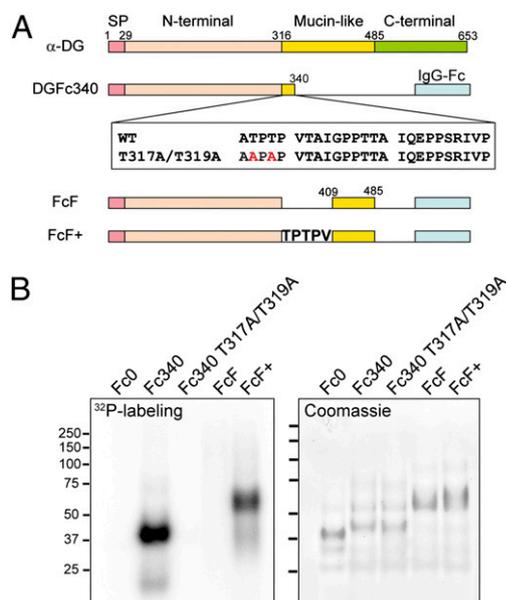
Fig. 3. The N-terminal region of the mucin-like domain provides acceptor sites for phosphorylated *O*-mannose glycans. (*A*) Schematic representation of dystroglycan mutant proteins. (*B*) Phosphorylation of *O*-mannosyl glycans takes place in the N terminus of the mucin-like domain. Dystroglycan mutants were expressed in HEK293 cells. Cells were incubated for 1 h in medium containing ^{32}P and then were cultured in the normal growth medium for an additional 4 d. (*Left*) The Fc proteins secreted to the medium were collected using protein A beads. Incorporation of ^{32}P into DGFc proteins was evaluated using a phosphorimager. (*Right*) The SDS-polyacrylamide gel also was stained with Coomassie brilliant blue.

(Fig. S5). Further mutational analyses show that T317A/T319A mutations abrogated the ^{32}P-incorporation on DGFc340 and that insertion of the TPTPV sequence between DGN and the C-terminal half of mucin-line domain (DGFcF+) significantly restored ^{32}P incorporation (Fig. 3*B*). These results suggest that the closely localized Thr residues (Thr-317/319) function as direct acceptor sites for phosphorylated *O*-mannosyl glycans.

Closely Localized Thr-317/319 Residues Are Crucial for the Functional Expression of Full-Length Dystroglycan in Myoblasts. α-Dystroglycan has been reported to serve as a cell-surface receptor for several ECM proteins containing LG domains, and the binding between α-dystroglycan and ECM proteins appears to initiate further matrix-assembly processes of the ECM (2, 3, 21). To examine the significance of Thr-317/319 residues in dystroglycan's function as a laminin receptor, we expressed full-length or mutated forms (Fig. 4*A*) in dystroglycan-null myoblast cells by adenovirus-mediated gene transfer. Both Western blotting with antibody IIH6 and an LM OL demonstrated that mutations within the conserved N terminus of the mucin-like domain strongly reduced both IIH6 reactivity and laminin binding, despite the comparable expression levels of core α-dystroglycan and β-dystroglycan (Fig. 4*B* and Fig. S6). We further performed a cell-surface laminin-clustering assay on dystroglycan-null myoblasts expressing WT or mutated forms of dystroglycan. Immunofluorescence results showed that laminin consolidated into plaque-like clusters on the surface of cells expressing dystroglycan-WT as well as in WT control myoblasts. In contrast, no cluster formation was observed on the cells expressing mutant dystroglycan proteins lacking an intact TPTPV sequence (Fig. 4*C*). Taken together, these results demonstrated that the closely localized Thr-317/319 residues play a crucial role in coordinating the assembly of extracellular laminin on the cell surface.

Assessment of the Role of Closely Localized Thr Residues in Arenavirus Entry into the Cell. The α-dystroglycan–binding region of the Old World arenaviruses LCMV and LFV was mapped
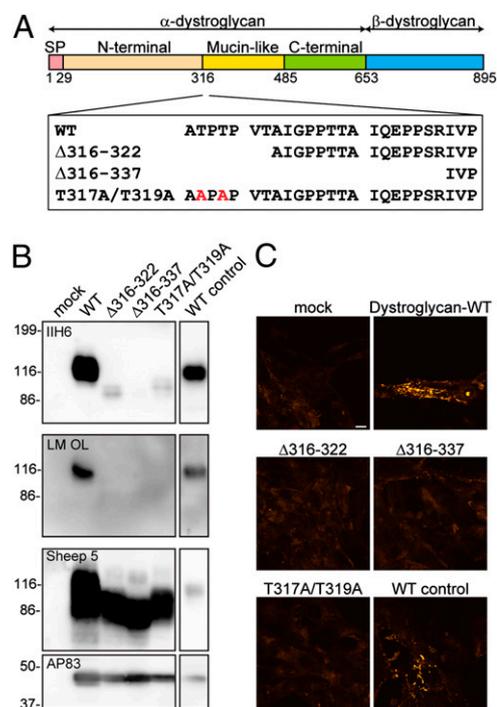

Fig. 4. The TPTPV sequence is crucial for dystroglycan's function as an ECM receptor in muscle cells. (*A*) Schematic representation of dystroglycan mutant proteins. (*B*) Expression, glycosylation, and laminin-binding activity of the dystroglycan mutant proteins. Wheat germ agglutinin-enriched preparations from dystroglycan-null myoblasts expressing WT or mutant forms of the protein and from WT control cells were analyzed either by Western blotting with an antibody against the α-dystroglycan core (sheep 5), β-dystroglycan (AP83), or IIH6 or by LM OL. (*C*) The TPTPV sequence is necessary for the formation of laminin clusters at the cell surface. Dystroglycan mutants were expressed in dystroglycan-null myoblasts using adenoviral vectors, and exogenous laminin-111 was added to the medium. Laminin cluster formation was detected by immunofluorescence and was found only in mutant cells that expressed dystroglycan-WT and in WT control myoblast cells. (Scale bar: 10 μm.)

previously to the first half of the mucin-like domain, and competitive binding analyses have indicated that the binding regions for arenaviruses and laminin overlap (11). To test the impact of the Thr-317/319 residues on arenavirus entry into the cell, a virus overlay protein-binding assay (VOPBA) was performed. VOPBA showed that both LCMV clone 13 and LFV bind to the DGFc334 and longer constructs (Fig. 5*A*). This result indicates that the first 18 amino acids, which contain the laminin-binding sites, also contain virus-binding sites.

We next examined whether the Thr-317 and -319 residues are necessary for dystroglycan to bind to both LCMV and LFV. To this end, adenoviral vectors were used to express full-length dystroglycan proteins (WT, T317A/T319A, or T328A/T329A) in dystroglycan-null ES cells. Lysates were prepared 48 h after infection and were subjected to affinity purification with Jacalin-conjugated beads to capture *O*-linked oligosaccharides. Comparable amounts of α/β-dystroglycan complexes were pulled down in both the mutants and in WT dystroglycan, as verified by Western blotting using a polyclonal antibody to β-dystroglycan, AP83 (Fig. 5*B*, β-dystroglycan). VOPBA showed that LCMV clone 13 and LFV bound to the T328A/T329A mutant less efficiently than to WT dystroglycan and that the binding was reduced further in the case of the T317A/T319A mutant (Fig. 5*B*, VOPBA LCMV clone 13 and LFV). In support of this observation, both viruses recognized the TPTPV sequence in the DGFcF+ protein (Fig. S7).

We also evaluated the ability of the dystroglycan mutants to function as cellular receptors for LCMV and LFV entry. After 48 h of adenovirus-mediated expression of dystroglycan proteins in
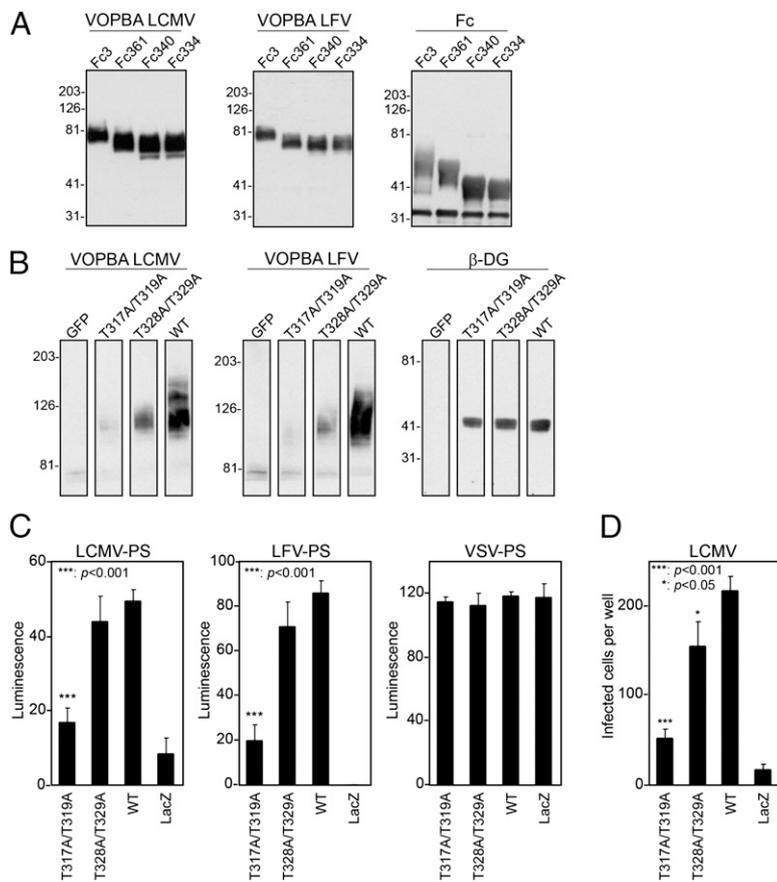
Fig. 5. Virus binding and cell entry into α-dystroglycan mutants expressed in dystroglycan-null ES cells. (*A*) Arenavirus binds to the region encompassing the first 18 amino acids of the mucin-like domain. DGFc proteins were coexpressed with LARGE in HEK293T cells. DGFc proteins purified from the culture medium were analyzed for laminin- and virus-binding activities. Shown are Western blotting with an anti-Fc antibody (Fc) and VOPBA using LCMV clone 13 (VOPBA-LCMV) and LFV (VOPBA-LFV). (*B*) Virus binding is impaired significantly in the dystroglycan-T317A/T319A mutant. Dystroglycan-T317A/T319A, -T328A/T329A, -WT, and GFP were expressed in dystroglycan-null ES cells, and a VOPBA was carried out. For VOPBA, 10^7 pfu/mL of LCMV clone13 (VOPBA-LCMV), or inactivated LFV (VOPBA-LFV) was applied. (*C* and *D*) Dystroglycan Thr-317 and -319 are crucial for efficient infection by arenaviruses. Dystroglycan-T317A/T319A, -T328A/T329A, -WT, and LacZ were expressed in dystroglycan-null ES cells, and the cells were infected with retroviral pseudotypes of LCMV (LCMV-PS), LFV (LFV-PS), or VSV (VSV-PS) (MOI = 10) or with live LCMV clone 13 virus (LCMV) (500 pfu per well). Statistical analysis was carried out using two-way ANOVA followed by a Bonferroni post hoc test with GraphPad Prism ($n = 3$; ***$P < 0.001$; *$P < 0.05$).

dystroglycan-null ES cells, the cells were infected with retroviral pseudotypes (PS) containing the glycoproteins derived from LCMV clone 13, LFV, or vesicular stomatitis virus (VSV), which does not use dystroglycan as a receptor (11). The recombinant retroviruses contain a luciferase reporter in their genome, and infection with the various pseudotypes was detected by luciferase assay. Infection with live virus (LCMV) was detected by immunofluorescence staining for the viral nucleoprotein, which is the earliest viral gene product. Reconstitution with the T317A/T319A mutant resulted in significantly reduced infection with both LCMV pseudotype (LCMV-PS) and LFV pseudotype (LFV-PS), indicating that T317A/T319A mutations impaired the function of dystroglycan as a receptor for LCMV and LFV (Fig. 5*C*, LCMV-PS and LFV-PS). The T328A/T329A mutant showed intermediate levels of cell entry by the virus and appeared to be partially functional as a virus receptor. Infection with VSV pseudotype (VSV-PS) was not affected by the expression of either mutated dystroglycan forms or WT (Fig. 5*C*, VSV-PS). The data obtained with the LCMV pseudotypes also were validated by infection with live LCMV clone 13 virus (Fig. 5*D*). Together, these data demonstrate that the posttranslational modification of dystroglycan at the closely localized Thr residues in the extreme N-terminal region of mature α-dystroglycan is crucial for the entry of the Old World arenaviruses LCMV and LFV into the cell.

Discussion

During posttranslational maturation, dystroglycan is modified extensively by glycosyltransferases (*N*-glycosylation, mucin-type *O*-glycosylation, and *O*-mannosylation) and proteases (processing of a single polypeptide into α- and β-dystroglycan and cleavage of DGN from the mature form of α-dystroglycan by a furin-like protease) (3). LARGE-dependent modification of phosphorylated *O*-glycosylation on α-dystroglycan is essential for its ability to bind to laminin and arenaviruses at the cell surface (18). Posttranslational modification of α-dystroglycan and its interactions with various ligands have been studied to under-

stand better the pathomechanisms of neuromuscular disease and severe LFV infections (4, 5, 11, 12, 16, 18, 20). However, a number of points regarding the molecular events involved in the expression of fully functional α-dystroglycan remain unclarified. In this report, we demonstrate that the first 18 amino acids in the α-dystroglycan mucin domain are necessary and sufficient to confer laminin-binding activity and arenavirus entry into cells. Moreover, the closely localized Thr-317/319 residues within a highly conserved amino acid sequence likely play an important role in LARGE-dependent glycosylation of α-dystroglycan.

The mucin-like domain of human α-dystroglycan contains 45 potential *O*-glycosylation sites, according to the prediction by the NetOGlyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc/). Our data demonstrate that single mutations of Thr-317 or Thr-319 nevertheless are sufficient to reduce α-dystroglycan's laminin-binding activity. Increases in both dystroglycan's glycosylation and laminin-binding activity following the TPTPV insertion between DGN and a Thr-rich region that is dispensable for functional glycosylation (DGFcF+) indicate that this sequence is crucial for the maturation to functional α-dystroglycan. One role of the TPTPV sequence may be to provide direct acceptor sites for phosphorylated *O*-mannosyl glycans as shown in Figs. 2 and 3. In addition, it is possible that modification of the TPTPV sequence promotes subsequent modification of other Thr/Ser residues located at other sites on α-dystroglycan. In either case, the TPTPV sequence may ensure that α-dystroglycan is modified efficiently and thus may contribute to the maintenance of membrane integrity in various tissues.

Our functional studies highlight the biological significance of the Thr residues within the first 18 amino acids of the mucin-like domain. The fact that DGN is cleaved by a furin-like protease during its posttranslational maturation (16) suggests that the TPTPV sequence localizes to the extreme N terminus of mature α-dystroglycan (Fig. S8). Thus, we hypothesize that DGN shedding ensures that α-dystroglycan is accessible to ECM ligands by

exposing the functional sugar chain. Indeed, assays measuring laminin clustering at the cell surface demonstrate that the Thr-317/319 residues are crucial for laminin organization on the cell surface. This result is consistent with the earlier in vivo finding revealing that functional glycosylation of α-dystroglycan is required for the proper localization of laminin/perlecan complexes in tissues of the central nervous system (4, 15). In lower organisms such as yeast, the glycosylation of transmembrane proteins is crucial for maintaining cell-wall integrity. In yeast, this function relies on a pathway that is maintained by several transmembrane proteins, such as the cell wall integrity and stress response component proteins and mating-pheromone-induced death 2 (Mid2p), all of which are known to be heavily glycosylated on their extracellular domains (22). Interestingly, a recent paper reported that the ability of Mid2p to function as a cell-wall mechanosensor requires *N*-glycosylation at an amino acid residue near the N terminus (23). It is tempting to speculate that the mechanism responsible for preserving membrane integrity may be conserved among species from yeast to human.

Glycans exert various biological roles via associations with specific glycan-binding proteins. Studies of glycan-binding proteins such as lectins indicate that multivalent interactions between clustered glycans and proteins bearing recognition domains for them are required for glycan-binding proteins to become high-affinity receptors; the affinities for single glycans tend to be low (for review, see ref. 24). In this report, we not only identify the positions of the Thr residues in α-dystroglycan that are important for this binding but also show that the closely localized Thr residues are necessary for maximal binding between α-dystroglycan and its ligands (Fig. S8). Although the enzymatic activities of LARGE remain to be characterized, one report suggests that an unidentified anionic modification on α-dystroglycan may supply the functional groups needed for ligand-binding activity (25). Interestingly, the binding of laminin α1 to α-dystroglycan depends on clusters of basic amino acid residues located on the surfaces of its LG domains (26). These findings suggest that the paired Thr residues, modified with anionic sugars, may participate in high-affinity multivalent interactions with the basic clusters on the LG-domain surface. Further work characterizing the LARGE-dependent modification will be necessary to unravel the mechanisms underlying the binding between dystroglycan and its ligands.

Previous studies demonstrated that LARGE-dependent glycosylation is essential not only for laminin binding but also for binding of LCMV and LFV to α-dystroglycan (9, 12). Our mutagenesis study reveals that for both viruses the first 18 amino acids in the mucin-like domain are necessary and sufficient to mediate this binding. In addition, we provide evidence that Thr-317/319 residues are required for α-dystroglycan's function as a cellular receptor for the Old World arenaviruses LCMV and LFV. The current hypothesis for the mechanism of virus entry is that arenaviruses with high affinity for α-dystroglycan are able to displace ligands, including laminin, from α-dystroglycan (2, 11). According to this model, small molecules with high affinity for arenaviruses (e.g., the hyperglycosylated TPTPV fragment) would be excellent leads for the development of novel therapeutic agents against LFV infection of humans.

In summary, we propose that the closely localized Thr-317/319 residues proximal to the LARGE-recognition domain serve as the direct targets of LARGE-dependent modification and that the modification at the extreme N terminus of the mucin-like domain is crucial for expression of the functional form of α-dystroglycan. Our findings provide insights into the mechanisms that lead to a functional, glycosylated dystroglycan product and thereby contribute to our understanding of α-dystroglycan–related pathogenesis of muscular dystrophy. Moreover, these findings provide a framework for establishing therapeutic strategies for treating both a variety of neuromuscular diseases and infection of humans by the hemorrhagic arenavirus LFV.

Materials and Methods

Biochemical Analyses. Biochemical analyses of dystroglycan mutants were carried out as described previously (16, 17). The in vitro ^{32}P-labeling assay on dystroglycan mutants was carried out as reported previously (18).

Arenavirus-Binding and Cell-Entry Assays. VOPBAs and cell-infection studies were carried out as described previously (11, 12).

Additional materials and methods are available in *SI Materials and Methods*.

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MEDICAL SCIENCES

Supporting Information

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SI Materials and Methods

Vector Construction for Expression of Recombinant Dystroglycan Proteins. Deletion mutants of α-dystroglycan fused to the constant region of immunoglobulin G (DGFc) were constructed by PCR. Some of the site-directed and deletion mutants were generated using a two-sided splicing method involving overlapping extension (1). PCR products were digested with KpnI/BclI and then inserted into the KpnI/BamHI sites of Ig-G1FcpcDNA3 (2). For DGFc mutants composed of the DGN, the TPTPV sequence, the fragment 409-485, and Fc (DGFcF+; IEGR, T317A, T319A, and T317A/T319A), PCR products were digested with HindIII and then inserted into the HindIII sites of pcDNA DGFcF+. The mutations were verified by nucleotide sequencing. For the generation of adenovirus vectors expressing dystroglycan mutants Δ316–322, Δ316–337, T317A, T319A, T317A/T319A, and T328A/T329A, the KpnI/XhoI fragments from the corresponding DGFc3 mutant were subcloned into pAd5RSVK-NpA (obtained from The University of Iowa's Gene Transfer Vector Core), as was the XhoI-XbaI fragment from an adenovirus vector that encodes dystroglycan-WT (3). The like-acetylglucosaminyltransferase (LARGE) expression vector was generated by subcloning the SmaI/NheI fragment from pAd5CMV5-LARGE (4) into the EcoRV/NheI site of the pIRESpuro3 vector (BD Biosciences). Detailed information about the constructions is available from the authors upon request.

Cell Culture. TSA201 and HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin. Mouse ES cells were cultured as previously reported (5). Transfection was performed using the FuGene 6 Transfection Reagent (Roche). Four days after transfection, DGFc proteins secreted into the culture medium were purified using protein A beads as described previously (6). When LARGE was coexpressed, the culture medium was harvested 2 d after transfection. The dystroglycan-null myoblast cell line was established as previously described (7).

Antibodies. The monoclonal antibody against the glycosylated form of α-dystroglycan (IIH6) and also the polyclonal antibody against β-dystroglycan (AP83) were characterized previously (6, 8). The polyclonal antibody against the core form of α-dystroglycan (sheep 5) is from sheep antiserum that was raised against the whole dystrophin-glycoprotein complex and was purified using a full-length α-dystroglycan-human IgGFc fusion protein, DGFc5 (8). Biotinylated anti-human IgG was obtained from Vector Laboratories. Antibodies used for a virus overlay protein-binding assay (VOPBA) and cell-infection studies [monoclonal antibody against glycoprotein 2 (GP2): clone 83.6; and antibody against lymphocytic choriomeningitis virus nucleoprotein (LCMVNP): clone 113] were described previously (9).

Factor Xa Protease Treatment. DGFc proteins (DGFcF+, and DGFcF+ IEGR) were expressed in HEK293 cells, and the secreted proteins were isolated, using Protein A-agarose beads (Santa Cruz) at 4 d posttransfection. DGFc proteins on the beads were incubated with Factor Xa (10 µg/mL) (New England Biolabs) in cleavage buffer containing 100 mM NaCl, 20 mM Tris (pH 8.0), and 2 mM $CaCl_2$ for 4 h at room temperature. After beads were washed twice with buffer containing 150 mM NaCl and 50 mM Tris (pH 7.5), proteins retained on the beads were eluted with Laemmli sample buffer.

Adenoviral Gene Transfer to Dystroglycan-Null Myoblast Cells. WT and dystroglycan mutants were expressed in dystroglycan-null myoblast cells using adenoviral vectors at a multiplicity of infection (MOI) of 1,000. Cell lysates were prepared 4 d after transfection and were subjected to wheat germ agglutinin (WGA) bead affinity purification. WGA-bound glycoproteins were subjected to SDS/PAGE and were analyzed by Western blotting using polyclonal antibodies sheep 5 and AP83, as well as by laminin overlay assays (LM OL). The Laminin-111 clustering assay was carried out as reported previously (6, 7).

Virus Strains and Purification. Purified lymphocytic choriomeningitis virus (LCMV) stocks were produced, and titers were determined as described previously (10). Lassa fever virus (LFV) (strain Josiah) was grown in Vero-E6 cells in a Biosafety Level 4 (BSL-4) facility, polyethylene glycol precipitated, and γ-inactivated at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, according to the method described previously (11). Inactivation was verified by infection assay. Live LFV was handled in a BSL-4 laboratory at the Special Pathogens Branch of the CDC. Work with LCMV was performed in a BSL-2 laboratory at The Scripps Research Institute.

Recombinant Moloney murine leukemia virus (MLV) pseudotypes, LCMV clone 13, LFV, and vesicular stomatitis virus (VSV) were produced as described (12). Briefly, the packaging cell line GP2-293 (BD Biosciences) was transfected with the packable murine leukemia virus genome pLZRs-Luc-gfp, which contains a luciferase reporter gene and a GFP reporter and was provided by Gary Nabel (Vaccine Research Center, National Institute of Allergy and Infectious Diseases; ref. 13). The GPs of LCMV clone 13, LFV, and VSV were provided in trans by cotransfection with expression plasmids containing their full-length cDNAs. Cell supernatants were harvested 40 h after transfection and were concentrated by ultracentrifugation, and pellets were resuspended as described. Titers were determined by infecting VeroE6 cell monolayers with serial dilutions of each pseudotype. After 48 h, cells were fixed for 10 min in 2% (wt/vol) paraformaldehyde in PBS. GFP was detected using a rabbit anti-GFP polyclonal antibody (#3080; Chemicon) as described previously (9). Specimens were examined under a fluorescence microscope, clusters of GFP-positive cells were counted, and titers were calculated.

Arenavirus-Binding and Cell-Entry Assays. Virus overlay protein-binding assays (VOPBAs) were carried out as described previously (3, 9, 12) using α-dystroglycan–Fc fusion proteins prepared from transiently transfected HEK293T cells (3). Full-length dystroglycan including WT-, T317A/T319A-, and T328A/T329A-dystroglycan proteins were expressed in dystroglycan-null ES cells following adenovirus-mediated gene transfer (3). Jacalin-agarose beads were used to enrich overexpressed full-length dystroglycan proteins, and the proteins were eluted by boiling in SDS/PAGE sample buffer. The expression levels of dystroglycan proteins were analyzed by Western blotting, using a polyclonal antibody against β-dystroglycan. For VOPBA, 10^7 pfu/mL of LCMV clone 13 or of inactivated LFV was applied (9). Bound virus was detected using monoclonal anti-GP2 antibody (clone: 83.6), and ECL was carried out using a peroxidase-conjugated secondary antibody.

For cell-infection studies, dystroglycan-null ES cells plated in 96-well plates were infected with adenoviral vectors containing WT-dystroglycan, the indicated dystroglycan mutants, or a LacZ reporter (MOI = 10). After 48 h, the cells were infected with

retroviral pseudotypes (PS) of LCMV (LCMV-PS), LFV (LFV-PS), or VSV (VSV-PS) (MOI = 10) or with live LCMV clone 13 virus (500 pfu per well). After an additional 48 h, infection was quantified using the Bright-Glo luciferase reporter gene assay (Promega), as measured in a Berthold 96-well plate luminometer. Luminescence is expressed as fold-increase over that for uninfected controls. Live virus infection was quantified after 24 h by immunofluorescence staining for LCMVNP.

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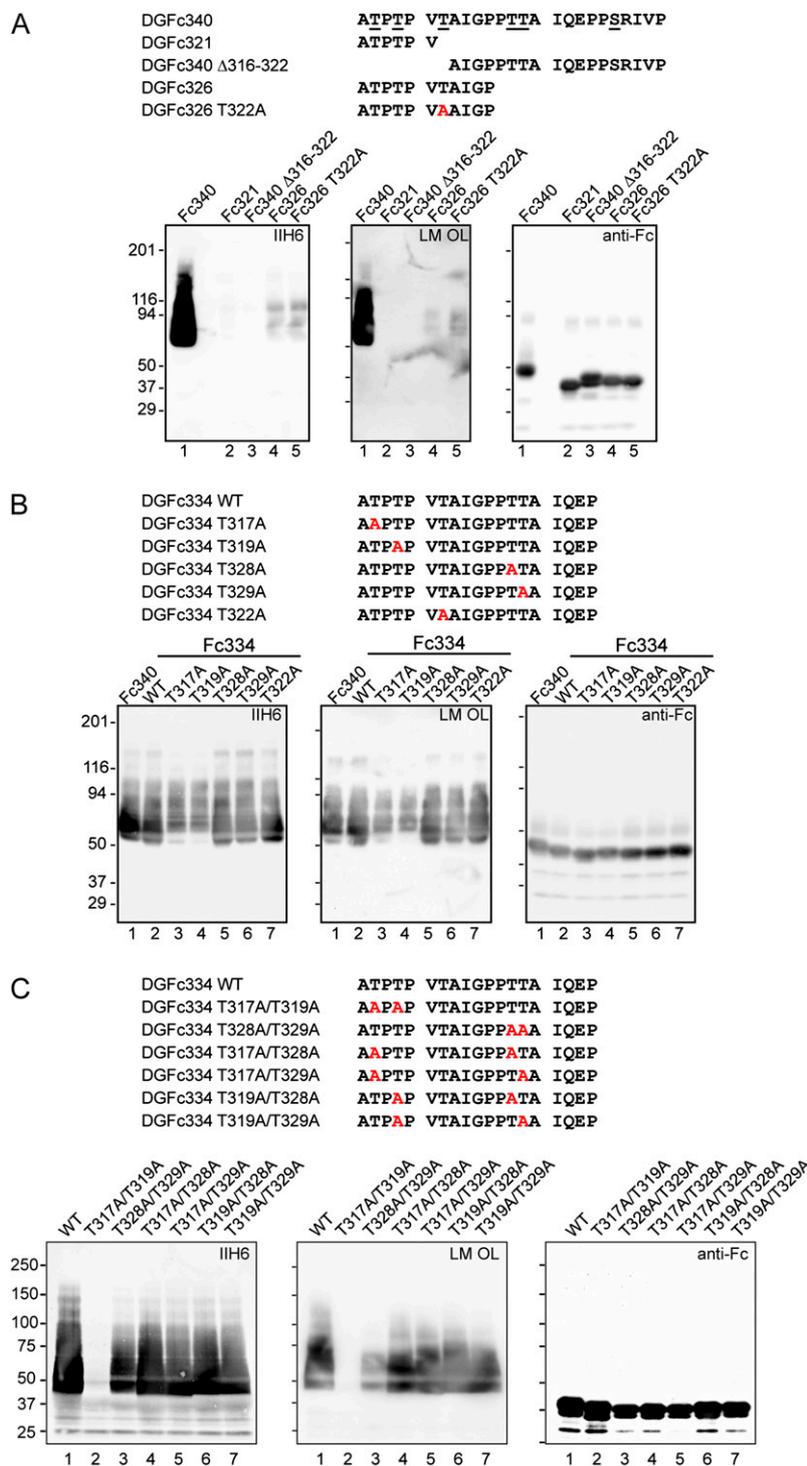
Fig. S1. Identification of residues crucial for functional glycosylation in the N-terminal region of the dystroglycan mucin-like domain. Western blotting and laminin overlays were carried out to assess the efficiency of binding and thereby the level of functional glycosylation. (*A*) The laminin-binding sites map to within the first 24 amino acids of the mucin-like domain. Fine-deletion constructs of DGFc340 were coexpressed with LARGE in TSA201 cells. The sequences of the deletion mutants are shown above the images of the blots. The six Thr/Ser residues (underlined) among the 24 amino acids were considered potential glycosylation sites. (*B*) Site-directed mutagenesis of the potential glycosylation sites in DGFc334. The effects of Ala replacement on the potential glycosylation sites were examined. The sequences of the deletion mutants are shown above the images of the blots. Residues replaced with Ala are indicated in red. (*C*) Double mutation of Thr-317/319 dramatically reduces functional glycosylation. The sequences of the tested deletion mutants are shown above the images of the blots. Residues replaced with Ala are indicated in red.
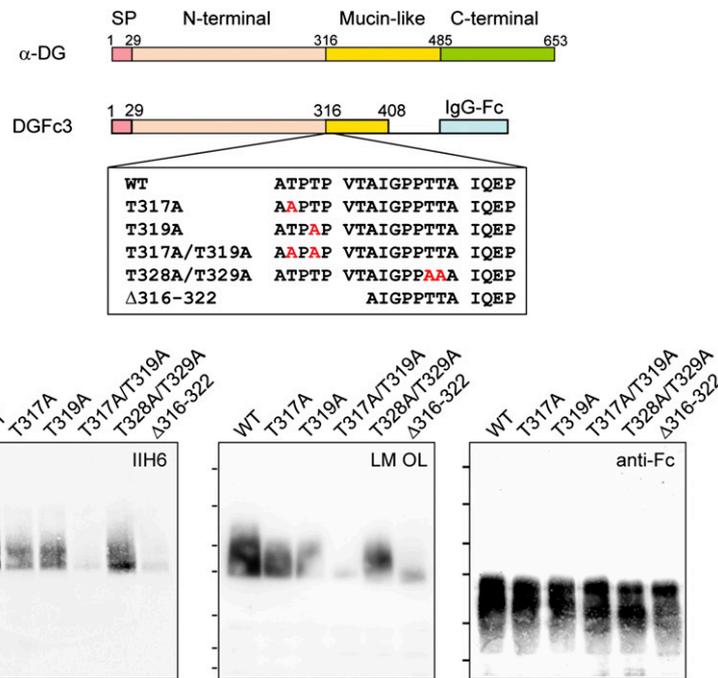
Fig. S2. Effects of the paired Thr-317/319 residues on the binding of laminin to the first half of the dystroglycan mucin-like domain (DGFc3). DGFc3 constructs in which Ala residues were replaced, individually or in pairs, were expressed in TSA201 cells, and the laminin-binding activities of these constructs were analyzed. These data show that Thr-317 and -319 residues are crucial for the dystroglycan glycosylation that is necessary for laminin binding.
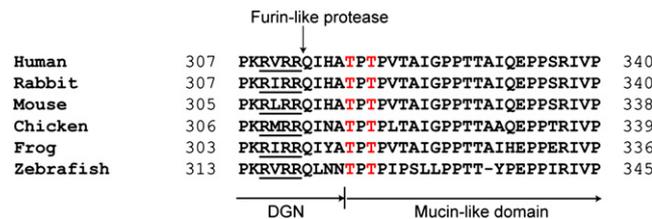
Fig. S3. Sequence comparison of vertebrate dystroglycan proteins. Amino acid sequences of dystroglycan around the furin-processing site and at the N terminus of the mucin-like domain are shown with the conserved furin cleavage motif (RXRR) in each underlined. The paired Thr residues located at the N terminus of dystroglycan's mucin-like domain are shown in red.
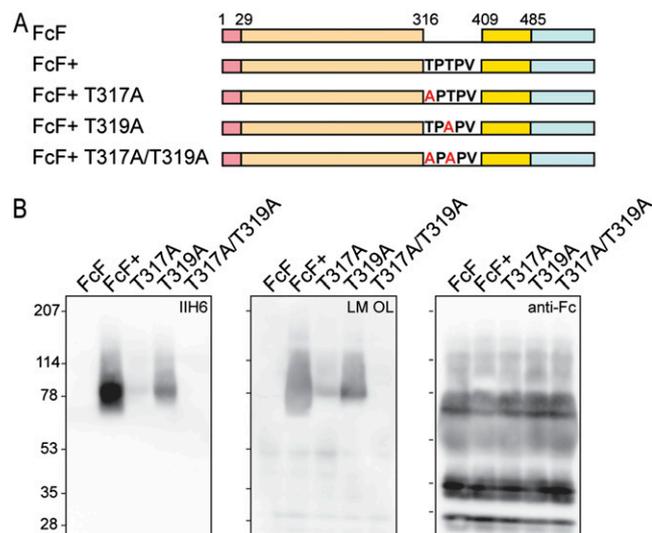
Fig. S4. Effects of individual Ala substitutions of the Thr^{317}ProThr^{319}ProVal (TPTPV) sequence of dystroglycan. (*A*) Schematic representation of DGFc mutant proteins. (*B*) DGFcF+ constructs with Ala replacements (T317A, T319A, and T317A/T319A) were coexpressed with LARGE in TSA201 cells, and functional glycosylation on the DGFc proteins was assessed with antibody to Fc, IIH6, or LM OL. Single mutation of either Thr-317 or Thr-319 reduced laminin binding, and double mutation of these residues resulted in a more pronounced reduction, even in the context of LARGE overexpression.

Fig. S5. The N-terminal region of the mucin-like domain, but not the α-dystroglycan N-terminal domain or the C terminus of α-dystroglycan, is required for phosphorylation of *O*-mannosyl glycans of α-dystroglycan. (*A*) Schematic representation of dystroglycan mutant proteins. (*B*) The α-dystroglycan N-terminal domain and the C terminus of α-dystroglycan are not required for phosphorylation of *O*-mannosyl glycans in the mucin-like domain. Fc-tagged dystroglycan mutants were collected from culture medium with Protein A-agarose beads and separated by SDS-PAGE. Incorporation of ^{32}P into the DGFc proteins was evaluated using a phosphorimager. When DGFc2 (the α-dystroglycan N-terminal domain fused to the Fc moiety) was used, a furin-like protease inhibitor (decanoyl-Arg-Val-Lys-Arg-chloromethylketone; CMK) was added to the medium to prevent cleavage of the α-dystroglycan N-terminal domain from the Fc (1).

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Fig. S6. Effects of individual substitution at residues Thr-317 or Thr-319 on dystroglycan's functional glycosylation in dystroglycan-null myoblasts. (*A*) Schematic representation of dystroglycan mutant proteins. (*B*) Expression, glycosylation and laminin-binding activity of the dystroglycan mutant proteins. WGA-enriched preparations from dystroglycan-null myoblast cells expressing WT or each mutant dystroglycan were analyzed by Western blotting with sheep 5, AP83, or IIH6 or by LM OL.

Fig. S7. The TPTPV sequence is important for binding of arenaviruses to dystroglycan. (*A*) Schematic representation of dystroglycan mutant proteins. (*B*) Arenavirus-binding activity of the DGFc mutant proteins. DGFc proteins (DGFc3, DGFc321, DGFcF, and DGFcF+) were coexpressed with LARGE in TSA201 cells. Protein A-agarose was used to enrich for proteins secreted into the culture medium, and the bound fractions were analyzed by VOPBA using LFV and LCMV clone 13 (LCMV).

Fig. S8. Proposed models for the roles of Thr-317/319 residues in the maturation and function of α-dystroglycan. (*A*) During the posttranslational modification of α-dystroglycan, *O*-mannosyl glycans of α-dystroglycan are phosphorylated in the Golgi apparatus (1). The interaction between the α-dystroglycan N-terminal region (DGN) and LARGE is crucial for post-phosphoryl modification of α-dystroglycan (2). In this study, we demonstrate that the Thr-317/319 residues are essential as direct LARGE-dependent glycosylation sites. The DGN is cleaved by a furin-like protease during translocation of α-dystroglycan to the sarcolemma (3). (*B*) The Thr-317/319 residues are located at the extreme N terminus of mature α-dystroglycan. Our results suggest that the maximal ligand-binding activity of α-dystroglycan is provided by (*i*) the proximity of the Thr-317/319 to the ligands including LG domains of extracellular matrix proteins and arenaviruses and (*ii*) the cluster of LARGE-dependent glycans.

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