A Method to Produce and Purify Full-Length Recombinant Alpha Dystroglycan: Analysis of N- and O-Linked Monosaccharide Composition in CHO Cells with or without LARGE Overexpression

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Jung Hae Yoon, Rui Xu, Paul Martin

Abstract

α dystroglycan (αDG) is part of the dystrophin-associated glycoprotein (DAG) complex, a series of cytoskeletal, transmembrane, and membrane-associated proteins that serve to link the extracellular matrix (ECM) surrounding individual skeletal myofibers to the intracellular F-actin cytoskeleton. Glycosylation and ECM protein binding to αDG are regulated by a number of genes that, when defective, give rise to congenital or limb-girdle forms of muscular dystrophy termed dystroglycanopathies. One such dystroglycanopathy gene is LARGE. Here, we describe a method to produce and purify full-length, furin-resistant, recombinant αDG from CHO cells and CHO cells overexpressing LARGE (CHO-LARGE). In addition, we analyze the O- and N-linked monosaccharide composition of such proteins. αDG purified from CHO-LARGE cells had increased molar content of xylose and fucose relative to CHO, while no significant changes were found in N-linked monosaccharides. Glucuronic acid could not be quantified by the methods used. These studies describe a method to produce and purify the milligram amounts of αDG needed for certain biochemical methods, including monosaccharide analysis.

Key words: Dystroglycan, muscular dystrophy, xylose, fucose, laminin, LARGE

Correspondence: Paul.Martin@nationwidechildrens.org

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Introduction

Dystroglycan serves an essential role in preserving muscle membrane integrity by mediating binding of extracellular matrix (ECM) proteins present in the basal lamina to the F-actin cytoskeleton. Dystroglycan is comprised of two protein subunits: α dystroglycan (αDG), an extracellular membrane-associated protein, and β dystroglycan (βDG), a transmembrane protein. αDG and βDG are created by post-translational cleavage from a single polypeptide precursor encoded by the dystroglycan (Dag1) gene. βDG is further cleaved such that the N-terminal third of the protein is removed by furin, while αDG can also be cleaved by matrix metalloproteinase. Once made, αDG binds to αDG in a very tight, non-covalent, complex. αDG serves as a major binding protein for ECM proteins including laminins, agrin, and perlecan, as well as for infectious agents including viruses and bacteria, while βDG, through its cytoplasmic domain, links this complex to F-actin-binding proteins, including dystrophin. Binding of many ECM proteins and infectious agents to αDG requires the proper O-linked glycosylation of αDG, which has been shown to contain core 1 O-glycans (Gal1,3GalNAc2-O) and O-mannosyl-binding glycans (Neu5Ac or Neu5Gc2,3Gal1,4GlcNAc2,1Man2-O) in tissues and on recombinant proteins.

Mutations in at least six genes lead to aberrant glycosylation of αDG in its mucin-rich region with O-mannosyl-linked tetrasaccharides of the type Neu5Ac/Neu5Gc2,3Gal1,4GlcNAc2,1Man2-O. In such disorders, termed dystroglycanopathies, loss of ECM binding correlates with loss of binding to glycan-dependent α dystroglycan monoclonal antibodies such as IIm216. In almost all such cases, αDG and βDG are still expressed at the sarcolemmal membrane of the skeletal myofiber, but αDG lacks important glycan structures needed for proper ECM binding, yielding muscular dystrophy which can be associated with brain and ocular malformations as well as cardiomyopathy. One such dystroglycanopathy gene is Large or LARGE, which has been recently described both as being required for synthesis of phosphate moieties on O-linked mannosyl glycans on αDG and also as a tandem 1,3glucuronic acid (GlcA) and 1,3xylose (Xyl) glycosyltransferase. LARGE may also have a therapeutic role in dystroglycanopathies, as its overexpression can stimulate increased glycosylation.
of ?DG even in disorders where other genes in the pathway are mutated. More recent work in transgenic mice also suggests that LARGE increases the apparent molecular weight of ?DG, but that this may result in reduced muscle specific force. The N-terminal domain of ?DG, which is normally cleaved off by furin, interacts with LARGE and, at least one mutation in the N-terminal domain of ?DG gives rise to Limb Girdle Muscular Dystrophy where LARGE-?DG interactions are reduced along with ?DG glycosylation. In addition, one recent report suggests that several amino acids just C-terminal to the furin cleavage on ?DG site bear the O-linked glycans required for laminin binding. To better understand the function of LARGE, we have analyzed the monosaccharide composition of full-length (furin-resistant) recombinant ?DG produced from CHO cells and from CHO-LARGE cells overexpressing LARGE.

**Results**

In order to be able to perform monosaccharide analysis, we wanted to develop a method to isolate and purify full-length recombinant a dystroglycan (?DG) in milligram amounts from transfected mammalian cell supernatant. We chose CHO cells for this purpose because many glycosylation mutants have been engineered with these cells. There were two major obstacles to this approach. The first was that the N-terminal third of ?DG polypeptide is almost stoichiometrically cleaved in skeletal muscle and many non-muscle cells by furin, a Golgi protease. Thus, the N-terminal third of the protein is normally missing from ?DG after purification from cells or tissues. Second, ?DG binds tightly, though non-covalently, to ? dystroglycan (?DG) in the muscle membrane. Thus, when the dystroglycan gene, which encodes both ?DG and ?DG, is overexpressed in cells, a heterodimeric ?/?DG protein complex is formed that co-localizes to the plasma membrane, allowing little ?DG secretion.

To surmount these obstacles, we first synthesized a cDNA encoding full-length ?DG with a stop codon at the end of the ?DG coding sequence. This allowed ?DG overexpression in the absence of ?DG. Because we wanted to enrich for full-length protein, we also placed a FLAG epitope tag at the N-terminus of the cDNA after the signal peptide. When this cDNA was transfected into CHO cells made to overexpress LARGE (CHO-LARGE), the recombinant secreted protein was cleaved, likely by furin, such that little to no full-length protein could be identified (Fig. 1). Addition of a cell-permeant furin inhibitor to ?DG-transfected-CHO-LARGE cells, however, led to expression of highly glycosylated (ca. 120-250kDa), FLAG-tagged, secreted protein (Fig. 1). To identify secreted ?DG, transfected cell supernatant was purified with anti-FLAG (M2) agarose. The ability to produce full-length ?DG was specific for furin inhibition, as addition of a plasmin inhibitor had no such effect (Fig. 1). Transfection of ?DG into C2C12 cells, which were then fused into myotubes, also allowed for secretion of full-length glycosylated recombinant ?DG protein, purified from transfected cell supernatant using anti-FLAG agarose, in several experiments (ca. 160-180kDa), but again this only occurred in the presence of a furin inhibitor (Fig. 1). Addition of the furin inhibitor to C2C12 myotube cultures, by contrast, caused no major change in ? dystroglycan expression (Fig. 1). Thus, in both muscle cells and in CHO-LARGE cells, a full-length FLAG-tagged ?DG protein could be secreted in relatively large amounts as long as furin was inhibited. We chose to utilize a recombinant protein with the FLAG tag at the N-terminus of the protein to enrich for recombinant full-length protein using anti-FLAG antibodies. Therefore, we did not make or utilize a recombinant protein with a FLAG tag placed at the C-terminus of the protein.

<table>
<thead>
<tr>
<th>CHO-LARGE (Secreted)</th>
<th>C2C12 Myotube (Secreted)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmin Inhibitor:</strong></td>
<td>- - - +</td>
</tr>
<tr>
<td><strong>Furin Inhibitor:</strong></td>
<td>- - + +</td>
</tr>
<tr>
<td><strong>αDG:</strong></td>
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<tr>
<td></td>
<td>75</td>
</tr>
<tr>
<td><strong>βDG:</strong></td>
<td>- - - - + +</td>
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<td><strong>Blot:</strong> IIH6</td>
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<td></td>
<td>75</td>
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<tr>
<td><strong>C2C12 Myotube (Cell Lysate)</strong></td>
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</tr>
<tr>
<td><strong>Blot:</strong> βDG</td>
<td><img src="image" alt="" /></td>
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<tr>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>

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cannot really be used to assay total glycosylation in this context, particularly for highly glycosylated glycoproteins where gel minimally changing the apparent molecular weight of delR312-?DG by SDS-PAGE. It is important to note that SDS-PAGE and did so equally well in protein purified from CHO-LARGE and CHO cells (Fig. 2). This was also the case for the but that does not block laminin binding, as IIH6 does furin cleavage mutants (not shown). Interestingly, VIA4-1, another glycan-dependent antibody that specifically recognizes ?DG, carbohydrate-dependent ? dystroglycan antibody and binding of recombinant laminin ?2 protein were dramatically increased on delR457, by contrast to delR79 and delR312, did not express much secreted protein at all. delR79/delR312 was mostly largely cleaved into a 40kDa fragment, while delR312 had minimal 40kDa fragment but high levels of 120-160kDa and 250 kDa protein. delR457, by contrast to delR79 and delR312, did not express much secreted protein at all. delR79/delR312/delR457 showed a pattern similar to delR312 but was more poorly expressed. Thus, the delR312 mutation was sufficient to inhibit the majority of N-terminal domain proteolysis on ?DG.

Next, we used molecular biology to eliminate each of the three potential furin cleavage sites in ?DG, RSFR (75-79), RIRR (309-312), and RTPR (454-457). This was achieved by deleting the final R at the furin cleavage site to create single amino acid deletions delR79, delR312 and delR457. Two double mutants (delR79/delR312 and delR312/delR457) and a triple mutant (delR79/delR312/delR457) were also made. Each of these constructs, or wild type (WT) ?DG, were transfected into CHO and CHO-LARGE cells. Recombinant proteins from the transfected cell supernatant were purified using anti-FLAG M2 agarose and analyzed for protein expression and fragmentation by SDS-PAGE with immunoblotting for the N-terminal FLAG tag (Fig. 2). Transfection of wild type (WT) ?DG predominantly produced cleaved N-terminal protein fragments, on average of about 40kDa, while little to no full-length protein was present. delR79 produced slightly more 120kDa protein than WT, but this mutant still was largely cleaved into a 40kDa fragment, while delR312 had minimal 40kDa fragment but high levels of 120-160kDa and 250 kDa protein. delR457, by contrast to delR79 and delR312, did not express much secreted protein at all. delR79/delR312 was mostly cleaved to 40kDa, while delR312/delR457 looked similar to the delR312. delR79/delR312/delR457 showed a pattern similar to delR312 but was more poorly expressed. Thus, the delR312 mutation was sufficient to inhibit the majority of N-terminal domain proteolysis on ?DG.

For all seven ?DG proteins studied, there was almost no apparent difference in the molecular weight profile by SDS-PAGE when comparing ?DG purified from CHO and CHO-LARGE cell supernatant (Fig. 2). Despite this, binding of the IIH6 carbohydrate-dependent ? dystroglycan antibody and binding of recombinant laminin ?2 protein were dramatically increased on delR312 ?DG purified from CHO-LARGE cells relative to CHO (Fig. 2). This was also the case for wild type protein and all other furin cleavage mutants (not shown). Interestingly, VIA4-1, another glycan-dependent antibody that specifically recognizes ?DG, but that does not block laminin binding, as IIH6 does (IIH6 or VIA4-1) or by laminin ?2 (LN?2) protein overlay.

A plasmid encoding a full-length cDNA for ? dystroglycan (?DG) with a FLAG epitope tag at the N-terminus was transfected into CHO-LARGE and C2C12 cells. Cells were incubated with cell-permeant inhibitor of furin or plasin, as indicated. ?DG protein was purified from cell supernatant using anti-FLAG agarose and blotted using anti-FLAG or anti-?DG (IIH6) antibody. Cell lysates made from transfected C2C12 myotube cultures were also blotted with antibody to beta dystroglycan (?DG).

Fig. 1: Inhibition of furin cleavage allows secretion of full-length recombinant alpha dystroglycan (?DG) from CHO-LARGE and C2C12 cells.

Fig. 2: Deletion of asparagine 312 allows for secretion of full-length recombinant ? dystroglycan (?DG) from CHO and CHO-LARGE cells.

For all seven ?DG proteins studied, there was almost no apparent difference in the molecular weight profile by SDS-PAGE when comparing ?DG purified from CHO and CHO-LARGE cell supernatant (Fig. 2). Despite this, binding of the IIH6 carbohydrate-dependent ? dystroglycan antibody and binding of recombinant laminin ?2 protein were dramatically increased on delR312 ?DG purified from CHO-LARGE cells relative to CHO (Fig. 2). This was also the case for wild type protein and all other furin cleavage mutants (not shown). Interestingly, VIA4-1, another glycan-dependent antibody that specifically recognizes ?DG, and did so equally well in protein purified from CHO-LARGE and CHO cells (Fig. 2). This was also the case for the del312/del457 double mutant (not shown). Thus, LARGE overexpression dramatically increased laminin binding, despite minimally changing the apparent molecular weight of delR312 ?DG by SDS-PAGE. It is important to note that SDS-PAGE cannot really be used to assay total glycosylation in this context, particularly for highly glycosylated glycoproteins where gel
migration profiles are not uniform.

To more explicitly demonstrate lack of cleavage at the R312 site in the delR312 mutant, we made and purified an anti-peptide 3DG polyclonal antibody, DG2, to amino acids 298-312 (HIANKKPPLPKRIRR) in the dystroglycan protein coding sequence. This peptide contains the protein sequence just N-terminal to R312 furin cleavage site. We immunoblotted wild type (WT) and delR312 3DG protein purified from transfected CHO cell lysates and supernatants with this antibody (Fig. 3). While WT 3DG secreted from CHO cells produced only 40kDa, cleaved, N-terminal fragment, delR312 protein produced only protein of only 160kDa or greater, again showing inhibition of proteolytic cleavage.

We next developed a method to produce milligram amounts of purified FLAG-tagged delR312 3DG protein from transfected CHO and CHO-LARGE cell supernatant (Fig. 4). We used a continuous flow protocol to perfuse CHO or CHO-LARGE 3DG(dR312)-transfected cell supernatant through packed M2 (anti-FLAG) agarose affinity columns. Once columns were loaded, they were washed and the FLAG-tagged protein periodically eluted with 2M MgCl2. We produced and purified 3DG(dR312) protein from about 1.5L of cell culture supernatant by this method, doing two such preps each for delR312 3DG-transfected CHO and CHO-LARGE cells. After dialysis of eluted protein, we purified delR312 3DG further using Wheat germ agglutinin (WGA) agarose columns, followed by washing and elution with 0.3M N-acetyl-D-glucosamine (GlcNAc). Eluted protein (about 20mg from 1.5L) was extensively dialyzed to remove GlcNAc and salts prior to analysis.

Fig. 3: Detection of uncleaved alpha dystroglycan using an antibody specific for the R312 furin cleavage site.

DG2, an affinity purified antiserum to the 15 peptides immediately N-terminal of the R312 protein cleavage site, was used to immunoblot cellular and secreted wild type (WT) and delR312 3DG protein after transfection of CHO cells.

We produced and purified 3DG(dR312)
CHO cells or CHO cells stably overexpressing LARGE (CHO-LARGE) were transfected with a FLAG-tagged cDNA encoding delR312 δDG. Supernatant was continuously collected and applied to anti-FLAG antibody (M2) affinity resin, which was batch eluted in 2M MgCl2. After dialysis, protein was further purified over Wheat germ agglutinin (WGA) agarose and eluted with 0.3M N-acetyl-glucosamine (GlcNAc). After dialysis, delR312 δDG protein was concentrated and studied.

The identity of delR312 δDG was first confirmed using silver staining and Western blotting (Fig. 5). Analysis of 5 micrograms of post-purified protein showed silver staining of high molecular weight material at 160-250kDa consistent with native δDG in both CHO- and CHO-LARGE-purified material. In addition, CHO cell material had increased expression of several lower molecular weight bands, at 72kDa and 60kDa, relative to CHO-LARGE. The 72kDa co-migrated with a FLAG-positive band and the 60kDa band co-migrated with a band blotted by an antibody to the extreme C-terminus of δDG (ns). Thus, it is possible that these lower molecular weight bands represent cleaved fragments of δDG. Immunoblotting of post-purified material with antibody to FLAG showed protein bands from 160kDa to 250kDa for both CHO and CHO-LARGE material. Thus, as before, the glycoforms of δDG present in post-purified material appeared to be the same molecular weights in both CHO- and CHO-LARGE transfected cells. The molecular weight profile was roughly equivalent in profile between CHO and CHO-LARGE, though silver staining of gradient gel-separated material showed a slight apparent difference in the migration of high molecular weight (160-250kDa) material (Fig. 5).
The identity of delR312 αDG from CHO and CHO-LARGE was also determined on 3 micrograms of sample by trypsin digestion followed by LC-MS/MS analysis. An example of one such sequenced peptide is shown in Fig. 6. Dystroglycan was the major protein identified by LC-MS/MS, with only bovine pancreatic trypsin inhibitor, which was added during purification, as a potential major contaminating protein. Bovine pancreatic trypsin inhibitor has no glycans that would interfere with subsequent αDG glycan analysis. Several other non-specific bands due to the preparation method, including trypsin and keratin, were also present (ns). Six common tryptic dystroglycan peptides were identified in CHO- and CHO-LARGE-purified delR312 αDG, at less than 5% false-detection rate (FDR), as follows: VTIPTDLIGSSGEVIK (aa80-95), LVPVVNR (aa227-234), LGCSLNQNSVPDIR (aa262-275), KPPLPK (aa303-308), GGEPNQRPELK (aa490-500), and VDAWVGTYFEVK (aa506-517). The P values for identification of alpha dystroglycan in this material were $2.3 \times 10^{-9}$ for CHO- and $1.2 \times 10^{-14}$ for CHO-LARGE-produced protein. 4 unique peptides in N-terminal domain and 2 unique peptides in C-terminal domain were detected in all delR312 αDG preps. In addition, for delR312 αDG purified from CHO-LARGE cells, two more tryptic peptides were identified: VSTPKPATPSDSSATTTTR (aa429-448) and LREQQVLGEK (aa540-549). Thus, sequencing of tryptic peptides from αDG purified from both CHO and CHO-LARGE cells by LC-MS/MS suggested the presence of full-length αDG protein.
N-linked and O-linked glycans of delR312 ?DG purified from CHO and CHO-LARGE are shown in Figures 7 and 8, respectively. Trimethylsilylation of sugars released by hydrolysis gave multiple peak patterns by each sugar corresponding to tautomeric forms (?, and ?-anomers, and pyranoside and furanoside rings). Therefore, in the spectra of the TMS ethers there is no single fragment ion that discriminates between the different monosaccharide classes, but these and their relative stereochemistries are readily discriminated by their retention times. Glycosaminoglycans and monosaccharides from decorin in equine systemic proteoglycan accumulation (ESPA) by GC/MS were successful after 2hr hydrolysis followed by acetylation with pyridine/acetic anhydride in methanol, however, in this work, after the 16hr extended period of hydrolysis used to liberate glycan followed by acetylation, we were unable to detect glucuronic acid (GlcA). Thus, the use of a protocol did not allow for quantification of glycosaminoglycans.

Fig. 7: Chromatograms of monosaccharides from N- and O-linked glycans on delR312 ?DG after purification from CHO cells.

N-and O-linked monosaccharides were separated from del312 ?DG purified from transfected CHO cells and analyzed by gas chromatography of their constituent monosaccharides as their trimethylsilyl derivatives, confirmed by standards, some of which are indicated.
In the analysis of O-linked glycans, glucose was again present as a contaminant. Monosaccharide compositions from CHO and CHO-LARGE delR312 ?DG were similar, with the presence of Gal, GalNAc, GlcNAc, GalNAc-ol, NeuNAc and Man-ol identified in both samples. GalNAc and Man were also detected as addittols, suggesting that the released glycans were originally O-linked to the protein backbone via GalNAc or Man residues at the reducing end. In addition, small amounts of fucose and xylose were detected (see Table 2). The relative amounts of monosaccharides in CHO-LARGE relative to CHO were constant for a number of monosaccharides (1:2.1:6:1.0:0.8:0.8) for Gal, Man, Man-ol, GlcNAc, GalNAc, respectively, somewhat different for GalNAc-ol and NeuNAc (5.2 and 0.4, respectively), and very different for fucose and xylose (14 and 43, respectively). Of these, xylose, fucose, and O-GalNAc were the only changes to reach statistical significance (P<0.05) using a standard two-tailed t test between CHO and CHO-LARGE. The increase in fucose and xylose for delR312 ?DG from CHO-LARGE, relative to CHO, is based on their being only a very small amount of these monosaccharides in the CHO-purified material. Thus, even when increased in CHO-LARGE, fucose and xylose still only represented a small proportional fraction of the O-linked monosaccharides, about 5% each. Nevertheless, these data suggest a significant increase in fucose and xylose in the monosaccharide contents of O-linked monosaccharides on delR312 ?DG purified from CHO-LARGE cells relative to CHO. The total O-glycan content in the two samples, however, was not significantly different. O-linked glycans were 33.3%, relative to protein, in delR312 ?DG purified from CHO cells and 37.0% in delR312 ?DG purified from CHO-LARGE cells. O-mannose linked glycans were almost exclusively detected on delR312 ?DG from CHO and CHO-LARGE (Fig. 9). Mannitol, arising from O-linked mannose, represented 99±9% of O-linked sugar (as defined by GalNAcitol plus mannitol) on CHO cell delR312 ?DG and 94±17% on CHO-LARGE delR312 ?DG. Thus, delR312 ?DG showed a very high concentration of O-mannose in both cell types.
Table 2. Composition of O-linked monosaccharides on delR312 ?DG purified from CHO and CHO-LARGE cells.

Trimethylsialylated monosaccharides liberated from O-linked glycans were quantified by GC/MS using calibration curves with standards. Errors are SD for n=2 experiments per condition.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>CHO Mole%</th>
<th>CHO-LARGE Mole%</th>
<th>CHO-LARGE/CHO Mole% Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>19.8±0.8</td>
<td>16.6±0.4</td>
<td>0.8±0.1</td>
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<td>Gal</td>
<td>6.1±0.6</td>
<td>7.6±0.7</td>
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</tr>
<tr>
<td>Man</td>
<td>1.4±0.5</td>
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<td>1.6±0.4</td>
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<tr>
<td>Man-ol</td>
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<td>54.7±0.1</td>
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<td>GlcNAc</td>
<td>2.6±0.2</td>
<td>2.1±0.1</td>
<td>0.8±0.1</td>
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<tr>
<td>GalNAc</td>
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<td>NeuNAc</td>
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<td>Xyl</td>
<td>0.1±0.0</td>
<td>4.4±0.1</td>
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</table>
Creation of FLAG-tagged furin cleavage-resistant ?DG mutants

In order to generate furin-resistant ?DG, we used the following three oligonucleotides to individually eliminate the fourth R at the transcription site.

Materials and Methods

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each of the three potential furin cleavage sites, RSFR(76-79), RIRR(299-312), and RTPR(454-457):
delR79: 5'TTGGGCGCTCGTTTGTGACCATTCCAACAGATTTAATTGGC3'
delR312: 5'CCAAACGCTATCGACAGATCCATGCCCACCCAC3'
delR457: 5'CCACGCGGACACCCCCGTGCCACGGGC3'

Using QuickChange Multi Site Directed Mutagenesis kit (Stratagene, 200515, CA), we generated a total of six secreted forms of ?DG mutants, all of which ended with a stop codon after the last amino acid of ?DG and therefore lacked all of beta dystroglycan (DG), as in 28. Mutants were made with N-terminal FLAG tag in pCMV1-FLAG vector as follows: ?DG(delR79), ?DG(delR312), ?DG(delR457), ?DG(delR79/R312), ?DG(delR312/delR457) and ?DG(delR79/delR312/delR457). All mutations were confirmed by DNA complete cDNA sequencing.

Cell Culture

Chinese Hamster Ovary (CHO) cells were obtained from American Type Culture Collection (ATCC). CHO cells were grown in DMEM with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin. CHO-LARGE cells were made by transfecting CHO cells with a myc-tagged cDNA encoding full-length LARGE. The plasmid containing the cDNA encoding LARGE was a gift from Pamela Stanley, Albert Einstein University. This plasmid also contained a selectable IRES-neo cassette to allow selection of stable transfectants in G418. CHO-LARGE cells were selected and grown in 400 μg/mL G418, which led to cells that stably overexpressed LARGE protein, as previously described. C2C12 cells, also originally from ATCC, were grown as myoblasts in DMEM with 20% FBS and 1% streptomycin and penicillin. To make C2C12 myotubes, C2C12 cells were grown to confluence and then fused for 3-6 days in DMEM with 2% horse serum and 1% streptomycin and penicillin, as previously described. All cell lines were incubated at 37°C in 5% CO₂-air mixtures in sterile, humidified, tissue culture incubators. Cell-permeant furin inhibitor (Dec-Arg-Val-Arg-Arg-CMK (chloromethyl ketone), Calbiochem 344930) and plasmin inhibitor (H-D-Val-Phe-Lys-CMK, Calbiochem 627624) were added to cells at 50μM for the full 2 days post-transfection following the manufacturer's instructions.

Cell Transfection

C2C12 cells or CHO or CHO-LARGE cells were transfected with CMV1-FLAG-?DG or pCMV1-FLAG carrying different ?DG mutants using Effectene transfection reagent (Qiagen) at 50-60% confluency. Transfected cells were cultured for 2-3 days prior to subsequent feeding. For transfected C2C12 cells, cells were co-selected in 400μg/mL G418 based after co-transfection with a 10-fold lower amount of a plasmid containing a neomycin resistance gene (neo). Cells were then fused in low serum for 3-6 days to make myotube cultures, as previously described.

Protein Purification

Media (10 ml x 60 plates) from transfected cells was cycled on a continuous basis through a packed M2 agarose columns as follows: Two columns, one each for untransfected or transfected CHO or CHO-LARGE cells, were packed with absorbent (Anti-Flag M2 agarose, 2 ml) and connected to channels of a peristaltic pump (Minipuls 3, Gilson). Each column was supplied with media (45 ml) from CHO or CHO-LARGE cells that had been transfected with an expression plasmid encoding full-length, furin-resistant, ?DG (delR312 aDG) or media from mock-transfected control cells. Media were circulated through the columns at a flow rate of 0.15 ml/min overnight. The columns were washed with 45mL of buffer A (20mM Tris-Cl, pH 7.4, 100mM NaCl, 1:50 protease inhibitor cocktail (Roche, cOmplete Ultra tablets)) over 6 hours to remove unbound materials. The desired glycoproteins were eluted with 45 mL of Buffer B (20mM Tris-Cl, pH 7.4, 100mM NaCl, 2M MgCl₂, 1:50 protease inhibitor cocktail) using a continuous flow system overnight. Dialysis, using 10,000MW cut-off dialysis tubing (Thermo Scientific, 68100), was carried out in Buffer C (20mM Tris-Cl, pH 7.4 with 1:50 protease inhibitor cocktail). Proteins were subsequently purified further in Buffer A using Wheat Germ Agglutinin (WGA)-coupled agarose columns, with elution in buffer A with 0.3M GlcNAc, as before. Dialysis (10000MW snake-pleated, Thermo Scientific, 68100) was then repeated using buffer C.

SDS-PAGE, Silver staining and Immunoblotting

Proteins (2-10 μg total) were separated on 4-12% SDS-PAGE gels. Gels were processed for silver staining or immunoblotting. For silver staining, the gel was fixed overnight in 50% ethanol containing 5% acetic acid. On the following day, the gel was incubated in silver working solution (Silver stain kit, Thermo 24612) for 30 min and then further incubated in the reducing solution for 5 min. The gel was then washed in water and incubated in a stabilizer solution for 40 min. Alternatively, proteins separated by SDS-PAGE were transferred to nitrocellulose and analyzed by immunoblotting, as previously described. Membranes were blocked in Tris-buffered saline (pH 7.4, with reduced (100mM) NaCl for IIH6 blots) with 0.02% Tween 20 (TBST) and 5% milk followed by incubating with IIH6 (Upstate Biotechnology, Lake Placid, NY) (1:2500) or anti-FLAG M2 monoclonal antibody (Sigma, 1:10000). Membranes were washed in TBST, incubated in anti-mouse IgM (IIH6), anti-mouse IgG (M2), or anti-Flag M2 conjugated to horseradish peroxidase, washed, and developed using the ECL chemiluminescence method.

Sequencing of purified of ? dystroglycan by Nano-LC-MS/MS

The sample was lyophilized before being reduced in ammonium bicarbonate (50mM) containing DTT (10mM) at 55°C for 45
min, followed by carboxymethylation with iodoacetic acid (55 mM) at room temperature in the dark for 30 min. Reduced and carboxymethylated delR312 ?DG was digested with sequencing grade-modified trypsin (1:30, Promega, Madison, USA) at 37°C overnight, followed by lyophilization.

All MS/MS experiments for peptide identification were performed using an LTQ-MS (Thermo Finnigan, USA) equipped with a nano-ESI source. The lyophilized peptide mixtures were solubilized in formic acid (0.1%) and loaded by an autosampler (Agilent 1100, USA) onto reversed phase columns (100 ?m i.d. x 12 cm, Zorbax SB-C18 packing material). Solvent A consisted of H$_2$O/CH$_3$CN (95:5 v/v) + 0.1 % formic acid and solvent B of H$_2$O/CH$_3$CN (20:80 v/v) + 0.1 % formic acid. Samples (10mL) were eluted at ~200 nL/min (HPLC pump at 10 ?L/min, split open). The RP-LC gradient consisted of: 0 to 10 % B (0-1 min), 10-45 % B (1-95 min), 45 to 60% B (95-110 min), 60 to 100 % B (110-115 min), 100 % B (115-120 min), 100 to 0 % B (120-121 min), and 100 % A (121-150 min).

Data-dependent MS acquisition conditions were as follows: 1 MS scan (3 microscans averaged) and 1 MS$^2$ on the top 13 most intense peaks; dynamic exclusion was enabled at repeat count 2, repeat duration 45s, exclusion list size 150, exclusion duration 45s, and exclusion mass width 1.5 m/z; collision-induced dissociation (CID) parameters were set at isolation width 3 m/z, normalized collision energy 35%, activation Q 0.25, activation time 30 ms. Both spectra were obtained at a heated capillary temperature of 200°C and an ESI voltage of 2.3 kV.

The fragment mass spectra were searched against a non-redundant database (NCBI) that included a contaminant database, using BioWorks (3.3.1SP1: ThermoFisher). The database search parameters included: only fully tryptic fragments were considered for peptide matching, the number of allowed missed cleavage sites was 2, the peptide tolerance was 2 Da, the fragment ion tolerance was 1 Da, and the capability to match one peptide sequence to multiple references within the database was set at 20. The calculations of FDR for our results were not obtained by applying the databases (original database and decoy database) due to insufficient data distribution. Instead, FDR for peptide matches in SEQUEST were estimated using the reverse sequence database strategy with transitional threshold at $X_{corr} = 1.9$ for $z = 1$, $X_{corr} = 2.5$ for $z = 2$, $X_{corr} = 3.8$ for $z = 3$

$Laminin overlays$

The LG1-5 domains of the mouse laminin alpha2 were purified from transfected non-ionic detergent HEK293T cell lysates as previously described protocol$^{28}$. Laminin ?2 was used in blot overlays, again as previously described$^{33}$.

$Release of O-linked oligosaccharides$

delR312 ?DG, purified from CHO and CHO-LARGE cells (3mg each per experiment) and fetuin (1mg), as a control, were denatured in sodium phosphate buffer (50 mM, 200 µl, pH 7.5) containing SDS (0.5%) and DTT (0.04 mM) at 100°C for 10 min. The denatured samples were then diluted into non-ionic detergent-containing enzyme buffer treated with PNGase F (10 units from Flavobacterium meningosepticum, New England Biolabs, MA) at 37°C overnight following the manufacturer's instructions. Digestions were terminated by heating the reaction mixture at 100°C for 3 min and enzyme removed by filter centrifugation (MW cut off 10 KD, Ultracel YM-10, Amicon). The centrifugates were lyophilized for the further analysis.

$Release of O-linked oligosaccharides$

Residues remaining on the filter following PNGase F digestion were centrifuged on the filter (MW cut off 50 KD, Ultracel YM-50, Amicon) to separate PNGase F from O-linked ?-dystroglycan. Residues remaining on the filter were lyophilized. To identify the O-linked core sugars, the glycan chains were released from the protein by reductive ?-elimination. O-linked ?-dystroglycan from CHO or CHO-LARGE (3mg each) and fetuin as a control (1 mg), as well as inositol (5 µg) as an internal standard, were dissolved in NaOH (0.05 M)-NaBH$_4$ (1M, 0.5 ml), vortexed and sonicated quickly. The samples were incubated for 16 hr at 45°C and then cooled to room temperature. After incubation, excessive NaBH$_4$ was destroyed with 10 % acetic acid and samples were passed through column of AG 50W-X8 (H$^+$) to remove Na$^+$ ions, and boric acid was removed by repeated evaporations with methanol-acetic acid (9:1) at 45°C by N$_2$.

$Derivatisation of Monosaccharides$

Monosaccharide standards: xylose, fucose, glucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, glucosamine, arabinol, xyliol, fucitol, glucitol, galactitol, mannotol, N-acetylgalactosaminol, glucuronic acid and galacturonic acid, fetuin and inositol, as an internal standard, were obtained from Sigma. Methanolic HCl (0.5M, 800 µl) was added to the samples and standards, which were kept for 16 hrs at 80°C. Samples and standards were dried down under N$_2$ with anhydrous methanol (500 µl x 3). For trimethylsilylation, Tri-Sil (Pierce, 200 µl) was added and incubated for 30 min at 80°C. The mixture was concentrated to dryness with a stream of nitrogen and reconstituted in hexane (2 ml). The solution was filtered through a glass wool packed column and concentrated again. Samples reconstituted in hexane (1 µl) were analyzed by GC/MS. For acetylation, samples were added to anhydrous pyridine (50 µl) and acetic anhydride (50 µl) in methanol (200 µl) and incubated for 2 hrs at room temperature and then dried with N$_2$.

$Glycan composition analysis by Gas chromatography/Mass spectrometry (GC/MS)$

GC/MS analyses were carried out using a gas chromatograph (Trace GC Ultra, Thermo Finnigan) equipped with a 30 m x 0.25
mm I.D. DB-1 column (Film 0.25mm, Agilent) connected to a mass spectrometer (Polaris Q, Thermo Finnigan). The split-splitless injector and transfer line temperature were set to 220°C and 270°C, respectively. The analytical conditions were as follows: Initial temperature 60°C, ramped to 260°C at 20°C/min and kept at 260°C for 3 min. The operating MS conditions were positive mode, scan rate 1 scan s⁻¹ over the range m/z 50–650 and source temperature 200°C. EI (electron ionization) mass spectra were measured in the total ion-monitoring mode and peak area (TIC) data were used for quantitative determination via calibration curves using standards.

**Competing Interests**

The authors have declared that no competing interests exist.

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**APPENDIX 1**

**Abbreviations**

?DG, alpha dystroglycan; ?DG, beta dystroglycan; CHO, chinese hamster ovary; DAG, dystrophin-associated glycoprotein; ECM, extracellular matrix; WT, wild type; TIC, total ion chromatogram

**References**


