The effects of post-translational processing on dystroglycan synthesis and trafficking[1]

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Abstract Dystroglycan is a component of the dystrophin glycoprotein complex that is cleaved into two polypeptides by an unidentified protease. To determine the role of post-translational processing on dystroglycan synthesis and trafficking we expressed the dystroglycan precursor and mutants thereof in a heterologous system. A point mutant in the processing site, S655A, prevented proteolytic cleavage but had no effect upon the surface localisation of dystroglycan. Mutation of two N-linked glycosylation sites that flank the cleavage site inhibited proteolytic processing of the precursor. Furthermore, chemical inhibition of N- and O-linked glycosylation interfered with the processing of the precursor and reduced the levels of dystroglycan at the cell surface. Dystroglycan processing was also inhibited by the proteasome inhibitor lactacystin. N-linked glycosylation is a prerequisite for efficient proteolytic processing and cleavage and glycosylation are dispensable for cell surface targeting of dystroglycan.

Key words: Dystroglycan; Processing; Mucin; Glycosylation; Proteasome inhibitor

1. Introduction

Transport of proteins to the cell surface is a complex process involving glycosylation, folding and sorting. These processes are initiated in the endoplasmic reticulum (ER) and continue in the Golgi apparatus as the protein proceeds along the secretory pathway [1]. An elaborate system for protein quality control exists such that misfolded proteins are removed from the ER and degraded in the cytoplasm by the proteasome [2]. Defects in the post-translational processing of dystroglycan, a component of the dystrophin glycoprotein complex (DGC), are a common feature of some congenital muscular dystrophies (CMDs) [3,4]. Dystroglycan is processed by an as yet unidentified protease into two proteins, the transmembrane dystrophin-binding protein β-dystroglycan and the extracellular matrix receptor α-dystroglycan [5]. In addition to the proteolytic processing both α- and β-dystroglycan are glycosylated. α-Dystroglycan is glycosylated in a tissue-specific manner to produce a number of distinct glycoforms [5]. Glycosylation of dystroglycan is required for binding to a number of extracellular proteins such as laminin [6] and agrin [7,8] and has also been implicated in viral [9] and bacterial adherence [10].

Several forms of CMD are associated with secondary alterations in dystroglycan processing (for recent review see [3]). These diseases include, Fukuyama congenital muscular dystrophy (FCMD), Muscle–eye–brain (MEB) disease, congenital muscular dystrophy type 1C (MDC1C) and Walker–Warburg syndrome (WWS) [11–15]. Furthermore, the myodystrophy (*myd*) mouse mutant also has secondary abnormalities in dystroglycan processing [16]. Each of these disorders is caused by mutation in an actual or putative glycosyltransferase. Mutations in protein O-mannosyltransferase 1 (POMT1) that cause WWS are predicted to block the first step in O-mannosylation. POMGnT1 is mutated in MEB and encodes a glycosyltransferase that catalyses the addition of N-acetylglucosamine to a peptide linked mannose residue [12]. Thus POMT1 and POMGnT1 are thought to catalyse sequential steps in the synthesis of O-linked glycans on α-dystroglycan. Patients with FCMD and MDC1C have mutations in two homologous proteins, fukutin and FKRP, that are predicted to be phosphoryl ligand transferases and possibly glycosyltransferases [13,17]. Consistent with a role in protein processing, these putative enzymes are found in the Golgi apparatus [18].

Dystroglycan processing and maturation poses a number of challenges to the cell. Dystroglycan is a heterodimeric receptor that is cleaved into two polypeptides which are heavily glycosylated and self-associate at the plasma membrane. Given that secondary defects in dystroglycan processing cause different forms of CMD we determined whether post-translational processing was a prerequisite for dystroglycan synthesis and trafficking in vitro.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis

The construct encoding chick dystroglycan cDNA (ChDG, EMBL accession number AJ584830) has been described previously [18]. The programs NetOGlyc 2.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 Prediction (http://www.cbs.dtu.dk/services/NetNGlyc/) were used to predict potential O- and N-linked glycosylation sites [19]. Pfu Turbo DNA polymerase (Stratagene) was used to create

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[1] EMBL accession number AJ584830.

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mutations in ChDG as described previously [18]. All constructs were sequence-verified. The primers used are shown below with mutated nucleotides underlined: **DG655f**: 5′-G GAC ATC GCC AAG GGC GCG ATC GTG GTG GAA TGG; **DG655r**: 5′-CCA TTC CAC CAC GAT CGC GCC CTT GGC GAT GTC C; **DG556f**: 5′-TCG TGG GTC CAG TTC GCC AGC ACC AGC CAG C; **DG556r**: 5′-G CTG GCT GGT GCT GGC GAA CTG GAC CCA CGA; **DG642f**: 5′-G TTC GGC GAT AGG GCC AGC AGC ACC ATC AC; **DG642r**: 5′-GT GAT GGT GCT GCT GGC CCT ATC GCC GAA C; **DG662f**: 5′-GTG GTG GAA TGG ACC GCC AAC ACC CTG CCC; **DG662r**: 5′-GGG CAG GGT GTT GGC GGT CCA TTC CAC CAC; **DG730f**: 5′-GAC GGC AGG GTG ATC GCG GAG GCG ACG CC; **DG730r**: 5′-GG CGT CGC CTC CGC GAT CAC CCT GCC GTC; **DG733f**: 5′-GTG ATC TCG GAG GCG GCG CCG ACG CTG G; **DG733r**: 5′-C CAG CGT CGG CGC CGC CTC CGA GAT CAC; **DG735f**: 5′-GAG GCG ACG CCG GCG CTG GCG GCT GGG AAG; **DG735r**: 5′-CTT CCC AGC CGC CAG CGC CGG CGT CGC CTC.

2.2. Tissue culture, transfection and drug treatment

CHO, HEK and COS-7 cells were maintained and transfected as described previously [18]. Cells were treated with glycosylation inhibitors (10 μg ml^{-1} tunicamycin, TM (Sigma); 4 mM benzyl-GalNAc (Sigma) and glucosidase inhibitors (2 mM 1-deoxynojirimycin (Sigma); 50 μg ml^{-1} castanospermine (Sigma)). Lactacystin (Sigma) and leupeptin (Sigma) were added to the cells 24 h after transfection at final concentrations of 10, 50 and 20 μM, respectively. Cells lysates were prepared from 5×10^5 cells 48 h after transfection and approximately 50 μg was used for Western blot analysis as described previously [20].

2.3. Antibodies and immunocytochemistry

The following primary antibodies were used in this study: 43DAG 8D5 (Novocastra), mouse monoclonal specific to β-dystroglycan that detects expressed ChDG and endogenous β-dystroglycan in CHO, COS-7 and HEK cells. Anti-α/β-dystroglycan is a rabbit polyclonal antibody raised against a bacterially express fusion protein spanning the processing site of chick dystroglycan [18]. This antibody detects chick α- and β-dystroglycan but does not cross-react with endogenous dystroglycan in CHO, COS-7 and HEK cells. The α-dystroglycan-specific sheep antibody has been described previously [21]. Cells were processed for immunocytochemistry as described previously [18].

2.4. Flow cytometry

HEK cells stably expressing the ChDG cDNA were grown in culture for 24 h. The cells were then treated with inhibitors as mentioned above and grown for a further 24 h. Cells were detached from the tissue culture dish with Versene (Gibco, Life Technologies) and centrifuged at $500\times g$ for 3 min. Cells were incubated with the α/β polyclonal antibody diluted 1:100 with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) for 45 min at room temperature. The cells were washed with PBS/1% BSA, centrifuged as above, and incubated at room temperature for 45 min with Alexa 488-conjugated anti-rabbit IgG (Molecular Probes). Cells were washed again and resuspended in 300 μl PBS/1% (w/v) BSA. The fluorescence on the cell surface was assayed using a FACScan Flow Cytometer (Becton Dickinson).

2.5. Immunoblotting

Forty-eight hours after transfection cells were lysed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 5% (v/v) 2-mercaptoethanol. Proteins were separated by SDS–PAGE on either 4–15% gradient gels (BioRad) or 10% gels, transferred to a nitrocellulose membrane (Schleicher and Schuell) and probed with antibodies against dystroglycan as described previously [18]. Proteins were visualised using enhanced chemiluminescence (Pierce).

2.6. Enzymatic deglycosylation

Proteins were extracted from transfected cells in PBS (Sigma) containing 1% (v/v) Triton X-100. Twenty μg of protein extract was boiled for 10 min in denaturing buffer (0.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol) prior to enzymatic deglycosylation. Treatment with PNGase F (Sigma) and Endo H (Sigma) were performed according to the manufacturer's recommendations. Samples were used for immunoblotting as described above.

3. Results

3.1. Effect of proteolytic processing on dystroglycan

Dystroglycan is processed into two polypeptides in vivo and in vitro. Sequence analysis around the processing site for the dystroglycan precursor revealed remarkable sequence similarity to the processing sites for several proteins that contain SEA-modules (Fig. 1A). In common with dystroglycan these proteins are also decorated with O-linked glycans and remain associated with their respective cleavage partner at the cell surface (see [22] for review). To determine whether proteolytic processing was a prerequisite for cell surface localisation, the cleavage site of ChDG was altered from serine to alanine (S655A) by site-directed mutagenesis. This amino acid is invariant in all SEA-module-containing proteins that have an internal proteolytic cleavage site (Fig. 1A). The mutant dystroglycan was synthesised at normal levels and glycosylated, however the mutation completely blocked cleavage into the α- and β-subunits (Fig. 1B). CHO cells transfected with the S655A mutant construct expressed α-dystroglycan on the cell surface and were indistinguishable from cells expressing wild-type dystroglycan precursor (Fig. 1C). Punctate cell surface immunoreactivity was seen in all cell types (data not shown). These data show that proteolytic processing is not necessary for membrane localisation of α-dystroglycan.

3.2. Effect of glycosylation inhibitors on dystroglycan trafficking and processing

Expression of ChDG in CHO cells produces dystroglycan that is processed into three major proteins, β-dystroglycan (43 kDa), α-dystroglycan (160 kDa) and a 90 kDa immature form of α-dystroglycan [18]. In order to determine the N-linked glycosylation profile of dystroglycan, protein extracts were prepared from ChDG-transfected CHO cells, and incubated with PNGase F (cleaves N-linked glycans regardless of complexity) and Endo H (cleaves high mannose N-glycans). The molecular weight of β-dystroglycan was decreased in both cases (Fig. 2A), and the polypeptide generated by PNGase F was smaller than the Endo H product indicating that at least two N-linked sites on β-dystroglycan had been glycosylated. In chick dystroglycan these sites are located at residues N662 and N705, however only N662 is conserved across different species (see Fig. 4A). The relative molecular mass of α-dystroglycan was also decreased by PNGase F digestion indicating the presence of N-linked glycans (Fig. 2A). Endo H had no apparent effect on the relative molecular mass of α-dystroglycan (Fig. 2A).

To determine the effects of glycosylation on dystroglycan processing, CHO cells were transfected with the ChDG cDNA followed by treatment with inhibitors of glycosylation. Cells were treated with tunicamycin, which inhibits en bloc N-glycosylation, benzyl-GalNAc, which inhibits mucin-like O-linked glycosylation, deoxynojirimycin and castanospermine, which are inhibitors of ER glucosidase I and II. Tunicamycin treatment resulted in the selective loss of β-dystroglycan and a reduction in the level of α-dystroglycan (Fig. 2B). The data were confirmed with the β-dystroglycan-specific antibody 43DAG 8D5 that showed that endogenous and heterologous β-dystroglycan was lost following tunicamycin treatment (data not shown). A slight reduction in the size of α-dystroglycan was also observed following tunicamycin treatment confirming the presence of N-linked glycans. In

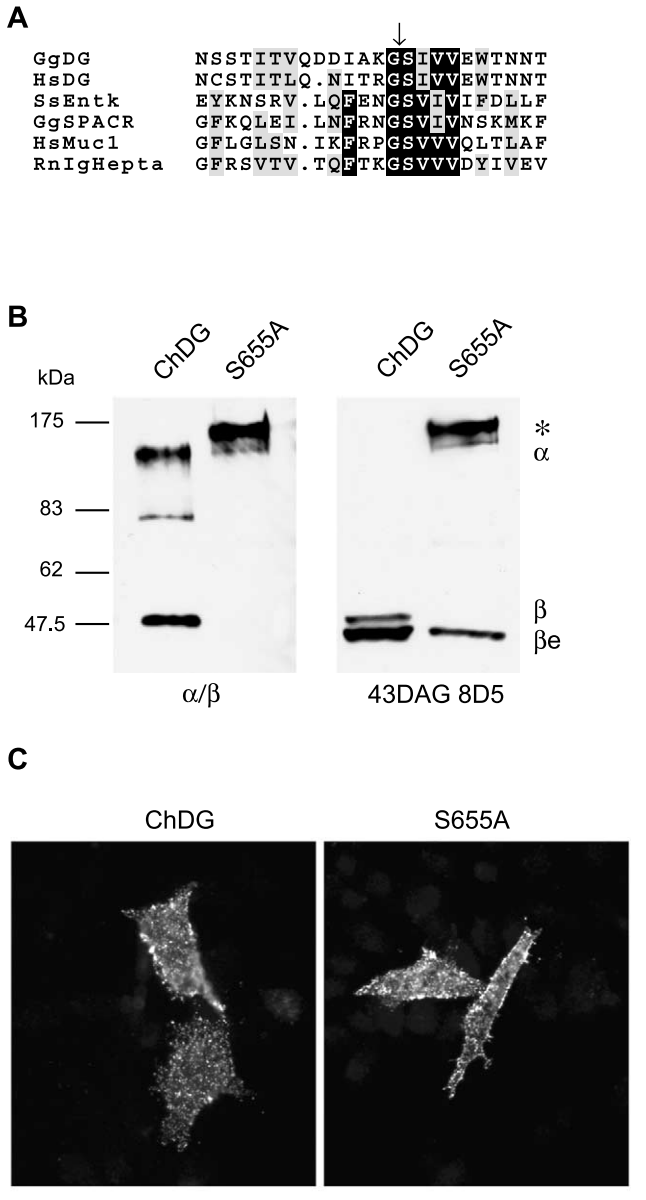

Fig. 1. The effects of proteolytic processing on dystroglycan trafficking. A: Sequence alignment of the processing site of dystroglycan precursor with similar regions in a subset of SEA-module containing proteins. GgDG, chicken dystroglycan; HsDG, human dystroglycan; SsEntk, pig enterokinase [34]; GgSPACR, chicken sialoprotein associated with cones and rods [35]; HsMuc1, human mucin-1; RnIgHepta, rat Ig-Hepta [36]. The position of the cleavage site between the conserved glycine and serine residue is indicated with an arrow. B: Extracts prepared from transfected CHO cells were blotted with antibodies against α-/β-dystroglycan (α/β) and β-dystroglycan (43DAG 8D5). The S655A mutant protein was uncleaved (asterisk) and detectable with both antibodies. The 43DAG 8D5 antibody also detected endogenous β-dystroglycan (βe). The 90 kDa protein in cells expressing ChDG corresponds to the immature form of α-dystroglycan. C: α-Dystroglycan was detected on the surface of CHO cells transfected with both wild-type (ChDG) and mutant (S655A) constructs. In control experiments no labelling was detected with 43DAG 8D5 that detects an intracellular epitope, indicating that paraformaldehyde fixation had not rendered the cells permeable to antibodies.

cells treated with benzyl-GalNAc, there was an approximate 2 kDa decrease in the relative molecular weight of β-dystroglycan indicating the presence of O-linked glycans and showing that the drug was active in CHO cells (Fig. 2B). This decrease was observed consistently over a range of benzyl-GalNAc concentrations (data not shown). Benzyl-GalNAc treatment also reduced the levels of α-dystroglycan but surprisingly had no apparent effect upon the relative molecular mass of α-dystroglycan (Fig. 2B). Deoxynojirimycin and castanospermine increased the abundance of the 160 and 90 kDa bands (Fig. 2B), and castanospermine treatment also caused a slight increase in the relative molecular mass of β-dystroglycan, presumably by inhibiting the action of ER glucosidases. Similar results were obtained in HEK and COS cells (data not shown).

Flow cytometry and immunocytochemistry was used to determine the steady state levels of α-dystroglycan at the cell

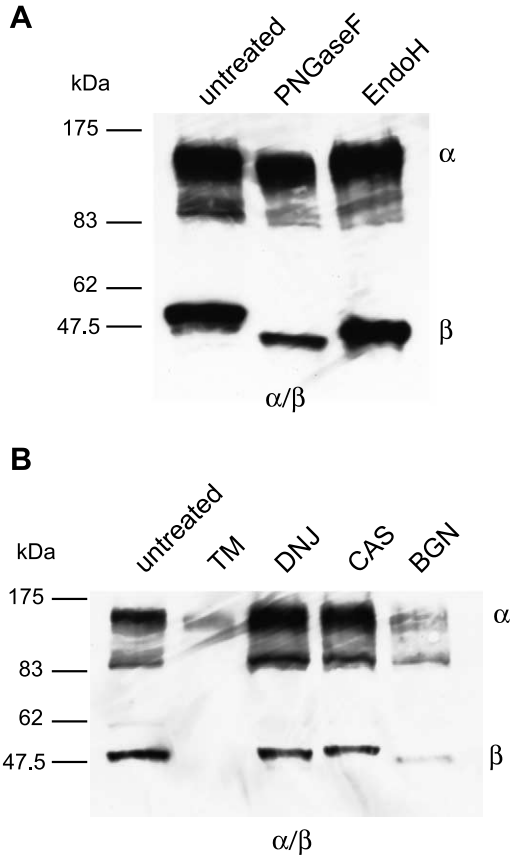

Fig. 2. Glycosylation profile and proteolytic processing of chick dystroglycan. A: Western blot of digested protein extracts derived from ChDG-transfected CHO cells, probed with the α-/β-dystroglycan antibody (α/β). PNGaseF and EndoH treatment reduced the size of β-dystroglycan, confirming the presence of N-glycans. PNGaseF also reduced the size of α-dystroglycan, demonstrating the presence of N-linked glycans. Proteins were resolved on 4–15% gradient gels. B: CHO cells were transfected with ChDG followed by incubation with inhibitors of glycosylation. Western blots of cell extracts were probed with an antibody against α-/β-dystroglycan (α/β). In cells treated with tunicamycin (TM) the 160 kDa α-dystroglycan band was deglycosylated with a reduced level of expression whereas β-dystroglycan was completely absent. The molecular weight of β-dystroglycan was reduced in benzyl-GalNAc (BGN)-treated cells. Deoxynorjirimycin (DNJ) and castanospermine (CAS) increased glycosylation of the 160 and 90 kDa products, and CAS increased the molecular weight of β-dystroglycan.

surface following treatment with the different glycosylation inhibitors. For these experiments we used a stable HEK cell line that expresses ChDG and transiently transfected CHO and COS-7 cells. Living, non-permeabilised cells were labelled with the sheep anti-α-dystroglycan antibody that detects extracellular epitopes on α-dystroglycan. In all cases surface labelling of dystroglycan was observed irrespective of the drug used. However, cells treated with tunicamycin and benzyl-GalNAc showed a reduction in fluorescence intensity indicating that less dystroglycan was present on the cell surface (Fig. 3). Similar results were obtained with transiently transfected CHO and COS-7 cells (data not shown). Castanospermine and deoxynojirimycin treatment caused a small increase in fluorescence intensity compared to the untreated control (Fig. 3) consistent with the increase in α-dystroglycan seen on Western blots (Fig. 2B). In control experiments no labelling was detected with antibodies raised against the intracellular epitopes showing that dystroglycan at the cell surface had the correct orientation and that only surface labelling was being observed.

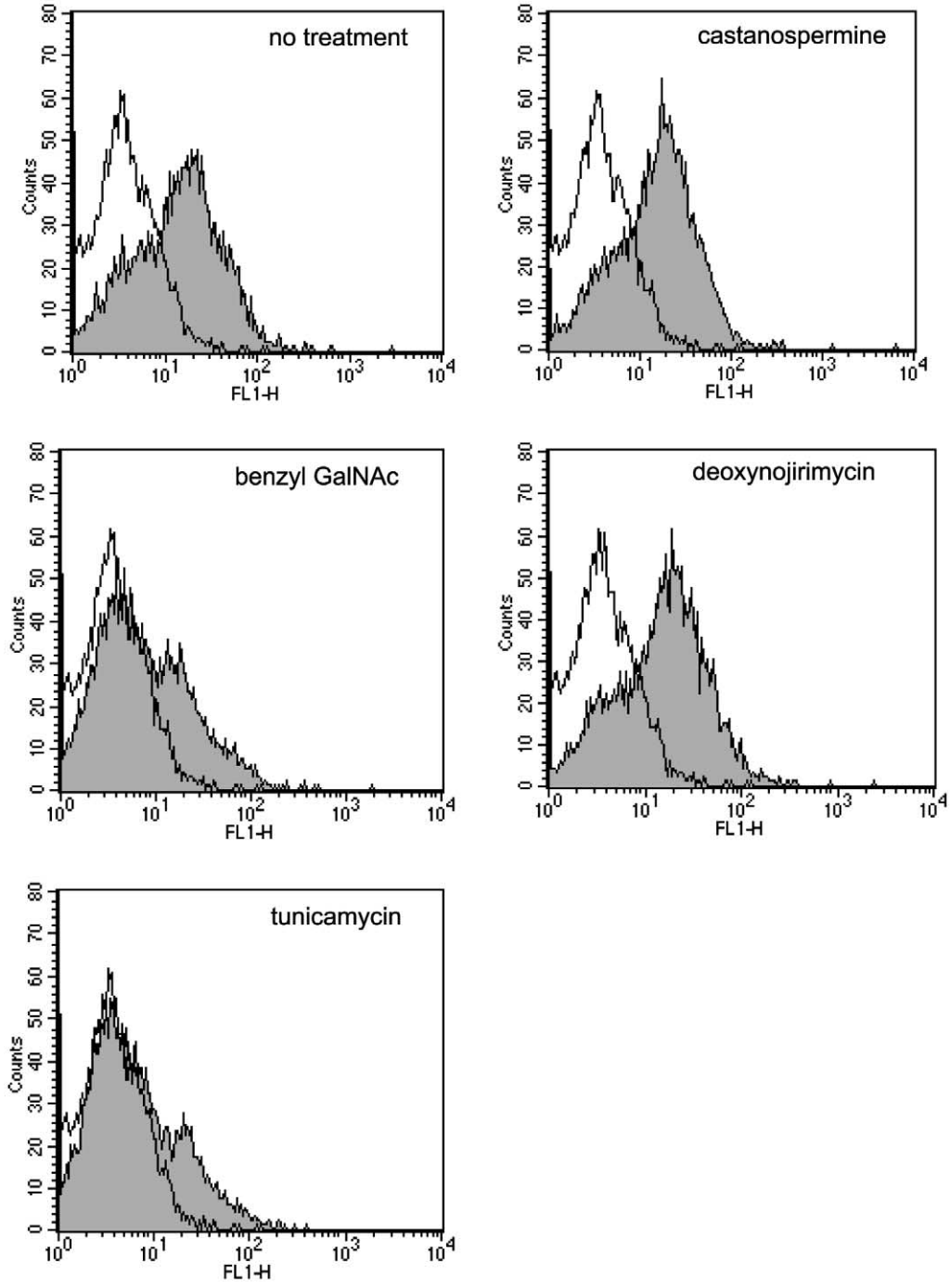

Fig. 3. Inhibition of N- and O-linked glycosylation does not prevent trafficking of α-dystroglycan to the cell surface. Flow cytometry was used to measure surface expression of α-dystroglycan on HEK cells stably expressing ChDG using an antibody against α-/β-dystroglycan. The primary antibody incubation step was omitted in controls. Tunicamycin and benzyl-GalNAc reduced the surface expression of dystroglycan.

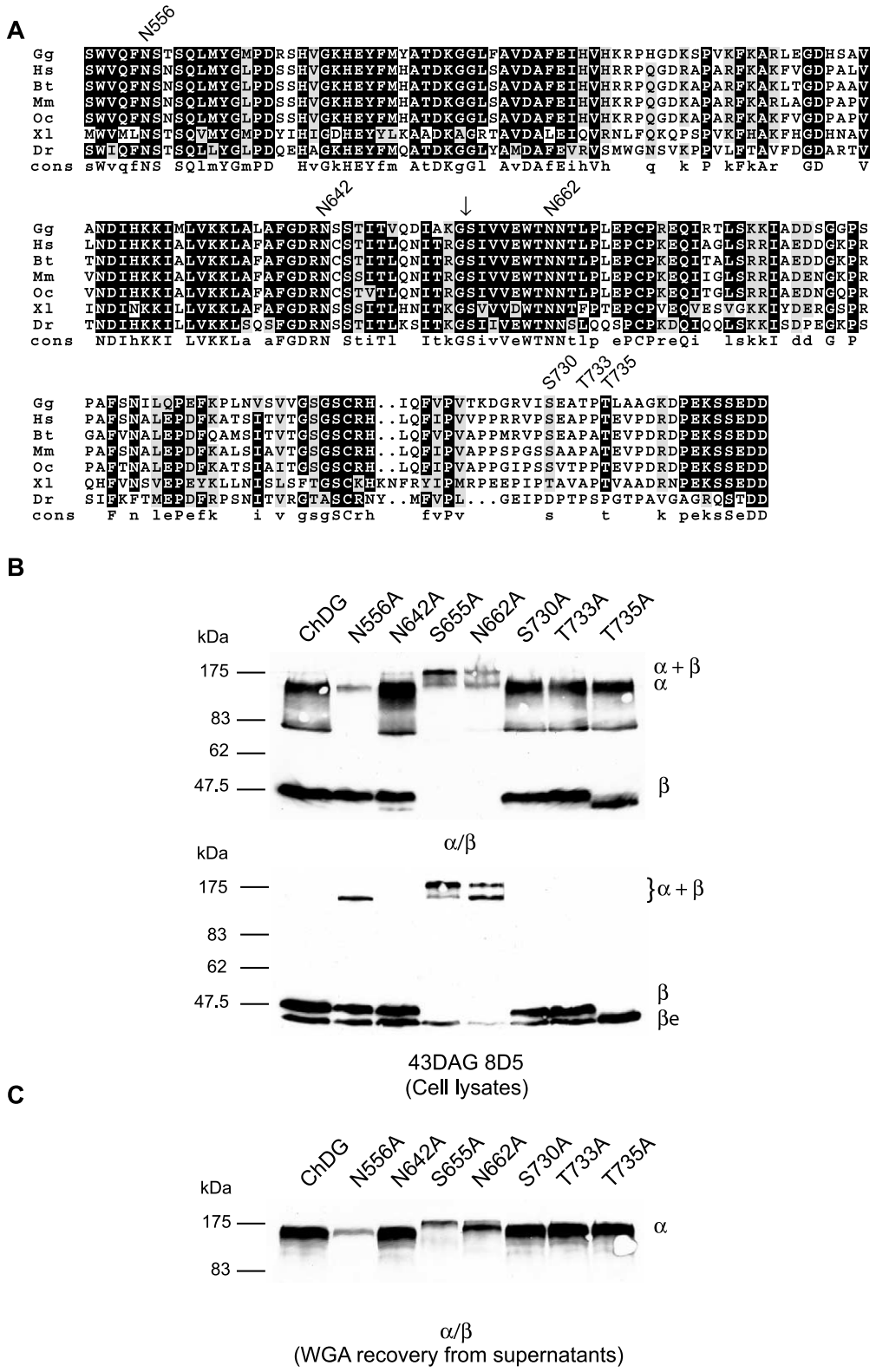

Fig. 4. Mutagenesis of putative glycosylation sites in ChDG. A: Sequence alignment flanking the processing site (arrow) of dystroglycan showing putative N- and O-linked glycosylation sites. Gg, *Gallus gallus*; Hs, *Homo sapiens*; Bt, *Bos taurus*; Mm, *Mus musculus*; Oc, *Oryctolagus cuniculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*. CHO cells were transfected with wild-type dystroglycan (ChDG) and mutations thereof, including the processing site mutant S655A. Cell lysates were analysed by SDS–PAGE and Western blotting with antibodies against α-/β-dystroglycan (α/β upper panel, B) and β-dystroglycan (43DAG 8D5 lower panel, B). Mutated amino acids, N556, S655, N662 and T735 altered the relative molecular mass of dystroglycan and/or the stoichiometry of the two proteins. By contrast, the mutations N642A, S730A and T733A did not alter the molecular weight of dystroglycan. Note that the mutants S655A and N662A also reduced the steady state levels of endogenous β-dystroglycan (βe). β-Dystroglycan produced by the T735 mutant co-migrates with endogenous β-dystroglycan. To determine whether the proteins were shed into the culture media, WGA-bound proteins from culture supernatants were probed with the α/β polyclonal antibody (C). Proteins with increased relative molecular mass were seen in cells expressing the mutants S655A and N662A. The identity of the proteins detected by each antibody is shown.

3.3. Effect of single glycosylation site mutants on dystroglycan processing

We have demonstrated a functional requirement for both N- and O-linked glycosylation for efficient processing and trafficking of dystroglycan. To specifically determine the molecular mechanism that controls glycosylation-dependent dystroglycan trafficking, putative glycosylation sites were altered by site-directed mutagenesis. The residues were chosen based upon their proximity to the proteolytic processing site, their prediction to be glycosylated and their cross-species conservation (Fig. 4A). These included two sites on α-dystroglycan (N556A and N642A) nearest to the cleavage site, and four sites on β-dystroglycan (N662A, S730A, T733A and T735A) on the extracellular side of the transmembrane region. The mutants were transfected into CHO cells to determine which sites were occupied by glycans and whether glycosylation at these sites was a requirement for proteolytic processing of the precursor. Mutation of T735A decreased the relative molecular mass of β-dystroglycan by approximately 2 kDa but did not alter the processing or stochiometry of α- and β-dystroglycan (Fig. 4B). The downward shift in relative molecular mass is identical to that seen with benzyl-GalNAc (Fig. 2B) suggesting that there is an O-linked glycan on β-dystroglycan at T735. No differences in the relative molecular mass of β-dystroglycan and the processing of the dystroglycan precursor were apparent in the mutants S730A and T733A (Fig. 4B). The mutant N662A behaved similar to the processing site mutant S655A (Fig. 4B). The N662A mutation completely abolished cleavage of the precursor protein resulting in lower levels of an uncleaved high molecular weight dystroglycan that was also detected with an antibody specific to β-dystroglycan (Fig. 4B). Interestingly synthesis of the mutants N662A and S655A interferes with the processing of endogenous dystroglycan such that lower levels of the protein are found in cell extracts from the uncleaved mutant compared to those in wild-type extracts or extracts prepared from the normally processed mutants (Fig. 4B).

Mutation of N556A in α-dystroglycan clearly reduced the levels of α-dystroglycan (Fig. 4B). This mutation is also associated with defective processing of the precursor because a high molecular weight protein is detected in the lysate with 43DAG 8D5 (Fig. 4B). By contrast, the mutation N556A in α-dystroglycan had no apparent effect on the processing of endogenous dystroglycan. Similarly, mutation of N642A had no obvious effect on dystroglycan processing or synthesis (Fig. 4B).

Several reports have shown that dystroglycan can be secreted from some cell lines [23–25]. To determine the effects of dystroglycan post-translational processing on this process, we tested two potential mechanisms for secretion or shedding of dystroglycan into the culture media: (1) Shedding is caused by dissociation of α- and β-dystroglycan at the membrane. (2) Shedding is due to limited proteolysis of α-dystroglycan. To distinguish between these possibilities we assayed WGA (wheat germ agglutinin) purified tissue culture supernatants from cells transfected with wild-type chick dystroglycan and the repertoire of mutants used in this study. Dystroglycan is detected in all the supernatants including those from the uncleavable mutants S655A and N662A. Anomalously high molecular weight dystroglycan (>180 kDa) was recovered from the supernatants of cell expressing the mutants S655A and N662A (Fig. 4C). The recovered protein is only detected by antibodies raised against α-dystroglycan and not with 43DAG 8D5, which detects an intracellular epitope on β-dystroglycan (data not shown). These data therefore suggest that proteolysis is the cause of α-dystroglycan shedding into the supernatant.

3.4. Effect of the proteasome inhibitor lactacystin on dystroglycan synthesis

Using site-directed mutagenesis and drug inhibition we have generated complementary data to show that N-linked glycosylation is a prerequisite for dystroglycan processing. The asparagine mutations that inhibit dystroglycan cleavage are associated with reduced synthesis of the precursor suggesting that the lack of N-glycosylation may cause protein misfolding and concomitant degradation by the proteasome. In addition, tunicamycin treatment reduces the level of α-dystroglycan and causes the selective loss of β-dystroglycan (Fig. 2B). To determine the fate of tunicamycin-treated dystroglycan, cells were transfected with ChDG and incubated for 24 h with tunicamycin alone and in combination with the proteasome inhibitor, lactacystin or with the inhibitor of lysosomal proteases, leupeptin. COS-7 cells were used because the combination of tunicamycin and lactacystin was toxic to CHO cells. Tunicamycin treatment resulted in the degradation of β-dystroglycan accompanied by a reduction in the amount of α-dystroglycan (Fig. 5A), as was the case in CHO cells (Fig. 2B). In cells treated with tunicamycin and lactacystin a 160 kDa band was detected using the β-dystroglycan-specific antibody (Fig. 5A). This suggests that tunicamycin treatment partially inhibits processing of dystroglycan and that the unprocessed and hypo-glycosylated dystroglycan is normally degraded via the proteasome pathway. Processed β-dystroglycan was not detected even with the addition of lactacystin or leupeptin (Fig. 5A), probably due to the requirement for N-linked glycosylation for efficient proteolytic processing (Fig. 4B).

Similar experiments were conducted on the N662A mutant (Fig. 5B). Addition of lactacystin increased the abundance of a lower molecular weight protein that was detected by both the α/β polyclonal and 43DAG 8D5 antibodies (Fig. 5B). In cells treated with lactacystin or lactacystin and leupeptin no endogenous β-dystroglycan could be detected suggesting that the proteasome inhibitor also interferes with the processing of the dystroglycan precursor (Fig. 5B). Accordingly, 43DAG 8D5 detects a doublet in cells treated with lactacystin that corresponds to the uncleaved endogenous and chick dystroglycan proteins. Addition of leupeptin to cells expressing the N662A mutant failed to increase the levels of dystroglycan in the lysate suggesting that the lysosome does not play a major role in the degradation of the transfected mutants (Fig. 5B). To determine whether lactacystin had a direct effect on dystroglycan processing, cells were transfected with wild-type ChDG and treated with the drug. Lactacystin treatment resulted in a dramatic reduction in the amount of β-dystroglycan and an increase in the levels of the 90 kDa immature form of α-dystroglycan (Fig. 5C). 43DAG 8D5 detected significant amounts of uncleaved dystroglycan in cells treated with lactacystin and also a clear reduction in the levels of processed endogenous and chick β-dystroglycan (Fig. 5C). Conversely, leupeptin had no apparent effect upon the steady state levels of dystroglycan. These data show that lactacystin blocks maturation of the dystroglycan precursor by inhibiting cleavage and possibly glycosylation.

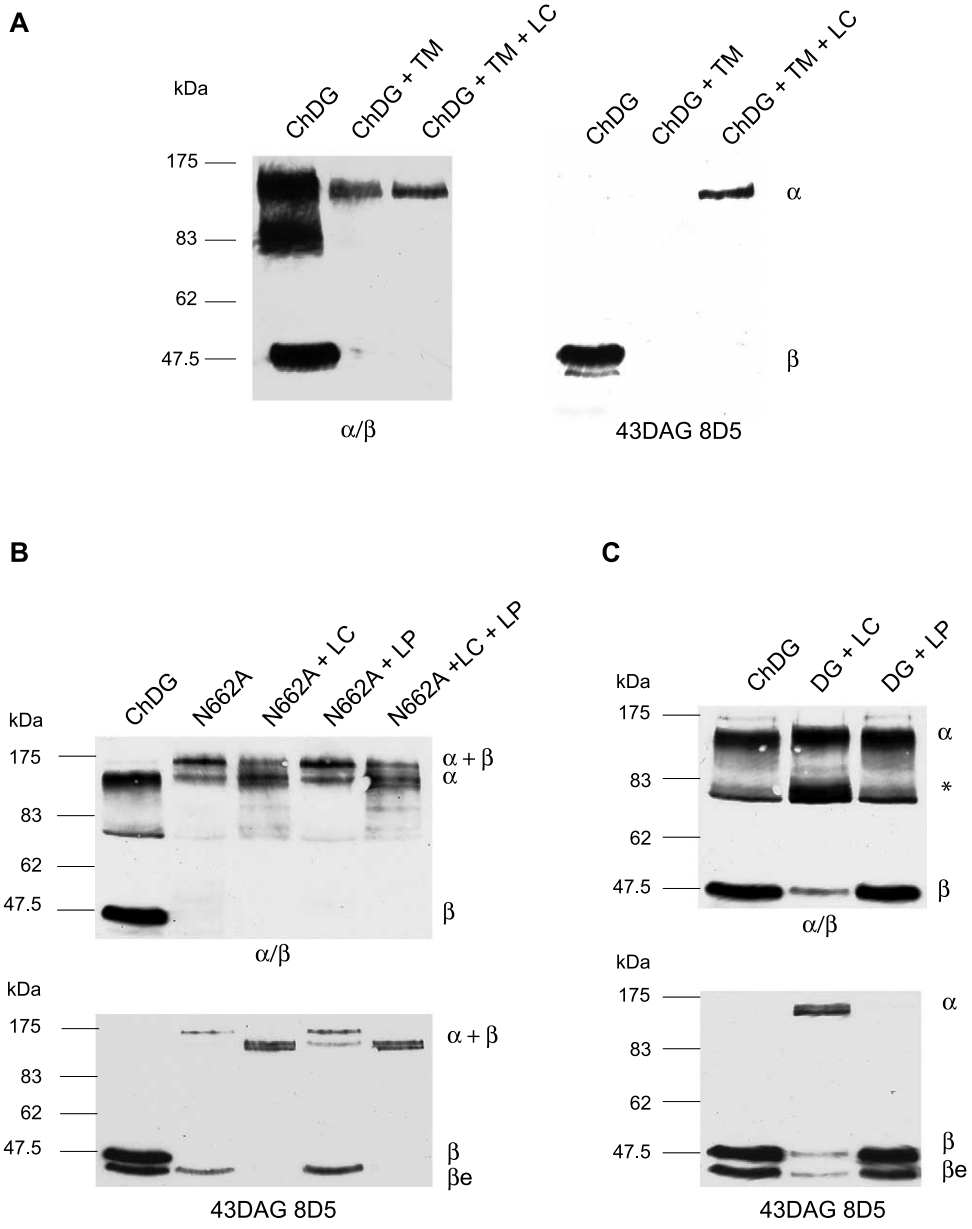

Fig. 5. The effect of lactacystin (LC) and leupeptin (LP) on dystroglycan processing. COS-7 cell extracts were prepared from cells transfected with ChDG and treated with tunicamycin (TM) and TM and LC (A). TM treatment causes the loss of β-dystroglycan and a reduction in the level of α-dystroglycan. Addition of LC increases the level of α-dystroglycan and stabilises the unprocessed precursor that can be detected with 43DAG 8D5. The effect of LC and LP on the steady state levels of the N662A mutant was assessed under the same conditions (B). LC increases the abundance of precursor protein that is detected with the α/β polyclonal antibody and as a doublet with 43DAG 8D5. LC treatment also blocked processing of endogenous dystroglycan as resulting in a high molecular weight protein detected by 43DAG 8D5 and no endogenous β-dystroglycan (βe). LP treatment had no apparent effect upon the mutant in the presence and absence of LC. LC also inhibits processing of wild-type ChDG (C) whilst LP had no apparent effect. The identity of the proteins detected by each antibody is shown.

4. Discussion

We have used an in vitro expression system to study the role of glycosylation and proteolytic processing on the synthesis and trafficking of dystroglycan. In this study we have presented complementary evidence showing that glycosylation and proteolytic processing of dystroglycan are dispensable for cell surface expression. We also show that N-linked glycosylation is important for proteolytic processing of dystroglycan.

To determine whether proteolytic processing was a prerequisite for cell surface expression a mutant dystroglycan that could not be processed was generated. This construct was effectively targeted to the surface of CHO cells despite the lack of processing. What then is the significance of the cleavage and re-association of the dystroglycan subunits? We have shown that the cleavage site, S655, is part of a conserved sequence found in SEA-modules that were first identified in a sperm protein, enterokinase and agrin (Fig. 1A) [22,26]. Importantly, this site is only conserved in SEA-domain proteins that are proteolytically processed. The SEA-module is found in a number of highly O-linked glycosylated membrane proteins including mucin-1. Wreschner et al., have proposed a mechanism whereby SEA-mediated cleavage generates a receptor–ligand partnership from the same protein for the pur-

pose of regulating signal transduction [22]. It is therefore possible that the binding of α-dystroglycan to one of its extracellular ligands elicits a signal through the SEA-module to β-dystroglycan. Recently Jayasinha et al. have shown that overexpression of an uncleavable dystroglycan mutant (mouse S654A) in transgenic mice causes muscular dystrophy associated with defective dystroglycan processing and glycosylation [27,28]. These data suggest that proteolytic cleavage is essential for faithful glycosylation and that the uncleavable mutant has the ability to inhibit the synthesis and glycosylation of endogenous dystroglycan [27,28]. Our findings described in this paper are in broad agreement with the behaviour of the S654A mutant in transgenic mice.

The glycans on α-dystroglycan are essential for binding to a number of extracellular matrix proteins including laminin [29]. Whilst glycosylation of α-dystroglycan is important for protein:protein interactions it is unclear whether the glycans are involved in processing and trafficking of the precursor protein. Furthermore, the role of glycosylation in β-dystroglycan is not understood. Using chemical inhibitors of glycosylation we have shown that tunicamycin and benzyl-GalNAc reduce but do not prevent dystroglycan trafficking to the cell surface. These data are in contrast to a study showing that tunicamycin inhibited trafficking of dystroglycan to the surface of CHO but had no effect on processing [25].

To examine the specific effects of glycosylation, we mutated conserved sites for N- and O-linked glycosylation. Mutations of the N-linked sites N556 and N662A flanking the SEA-module inhibit dystroglycan cleavage (Fig. 5). The mutation N662A completely abolishes dystroglycan processing into two peptides whereas N556A reduces the levels of α-dystroglycan (Fig. 5). Mutating the O-linked sites on β-dystroglycan did not have any obvious affect on dystroglycan processing other than a change in molecular weight (T735A mutant). These data show that N-linked glycosylation is a prerequisite for efficient proteolytic processing. These changes in dystroglycan processing could be a product of misfolding caused by the inhibition of N-linked glycosylation.

Proteins that misfold in the ER are translocated back to the cytosol and degraded by the proteasome, a process know as ER-associated degradation [30]. By adding the proteasome inhibitor lactacystin to dystroglycan-transfected cells that were pre-treated with tunicamycin, a population of uncleaved, partially processed dystroglycan was detected (Fig. 5A), indicating that misfolded dystroglycan is degraded by the proteasome. Similar results were obtained with the N662A mutant, again suggesting that misfolded protein was degraded by the proteasome (Fig. 5B). However, lactacystin was also found to inhibit the processing of wild-type dystroglycan (Fig. 5C). Whilst lactacystin is a potent proteasome inhibitor it has also been reported to inhibit the action of some proteases such as cathepsin A [31] and tripeptidyl peptidase II [32]. It is therefore possible that lactacystin inhibits the unidentified protease that processes the dystroglycan precursor. Interestingly, the proteasome inhibitor MG132 has recently been shown to rescue surface expression of the DGC in the *mdx* model of muscular dystrophy, showing that proteasome activity enhances disease progression in these mice [33].

In conclusion, we have demonstrated that glycosylation and proteolytic processing are not essential for targeting of dystroglycan to the cell surface. These data are important for understanding the mechanism of dystroglycan trafficking but are also clinically relevant because secondary alterations in dystroglycan processing are associated with several forms of CMD.

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