Enhanced laminin binding by α -dystroglycan after enzymatic deglycosylation

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Carbohydrate modifications are clearly important to the function of α -dystroglycan but their composition and structure remain poorly understood. In the present study, we describe experiments aimed at identifying the α -dystroglycan oligosaccharides important for its binding to laminin-1 and carbohydrate-dependent mAbs (monoclonal antibodies) IIH6 and VIA4₁. We digested highly purified skeletal muscle α -dystroglycan with an array of linkage-specific endo- and exoglycosidases, which were verified for action on α -dystroglycan by loss/gain of reactivity for lectins with defined glyco-epitopes. Notably, digestion with a combination of *Arthrobacter ureafaciens* sialidase, β (1-4)galactosidase and β -N-

acetylglucosaminidase substantially degraded Sia α 2-3Gal β 1-4GlcNAc β 1-2Man glycans on highly purified α -dystroglycan that nonetheless exhibited enhanced IIH6, VIA4₁ and laminin-1 binding activity. Additional results indicate that α -dystroglycan is probably modified with other anionic sugars besides sialic acid and suggest that rare α -linked GlcNAc moieties may block its complete deglycosylation with currently available enzymes.

Key words: α -dystroglycan, deglycosylation, laminin, lectin, muscular dystrophy, oligosaccharide structure.

INTRODUCTION

The dystroglycan complex is a component of the dystrophin-glycoprotein complex, which spans the sarcolemma of striated muscle cells and physically couples the actin cytoskeleton with the extracellular matrix [1–3]. The dystroglycan complex consists of α -dystroglycan, a highly glycosylated extracellular protein that binds to several extracellular ligands, and β -dystroglycan, a single-pass transmembrane protein that links cytoplasmic dystrophin with α -dystroglycan [3]. Both dystroglycan subunits are encoded by a single highly conserved propeptide that is proteolytically processed into α - and β -dystroglycan, which remain stably associated through non-covalent interactions [3]. Like muscles deficient in dystrophin or other core components of the dystrophin-glycoprotein complex [1,2], deficiency of the dystroglycan complex in skeletal muscle results in compromised sarcolemmal integrity [4,5]. Thus it is generally thought that one important function of the dystroglycan complex in skeletal muscle is to mechanically protect the sarcolemma against shear stresses imposed during muscle contraction.

O-mannosyl-linked oligosaccharides are clearly important for α -dystroglycan binding to its extracellular ligands as well as its function *in vivo* because mutations in glycosyltransferases that O-mannosylate α -dystroglycan result in loss of extracellular ligand binding activity and muscular dystrophy [3,6–9]. α -Dystroglycan is also modified by more generic N-linked glycans and sialylated core 1 oligosaccharides with the structure Sia α 2(3)Gal β (1-3)GalNAc [10]. However, most studies show that enzymatic removal of these glycans had no effect on α -dystroglycan binding to laminins [11], agrin [12] or neurexins [13]. At least two different O-mannosyl-linked oligosaccharides modify α -dystroglycan [14–16] but their specific role in extracellular matrix ligand binding is controversial [3]. In the present study, we attempted to identify α -dystroglycan oligosaccharides that are important for laminin-1 binding through digestion with various

combinations of linkage-specific endo- and exoglycosidases and reactivity with a panel of lectins of known carbohydrate specificities. The sialyl O-mannosyl glycan previously implicated in α -dystroglycan binding to laminin [14] was substantially degraded with a cocktail of three glycosidases, yet the laminin-1, monoclonal IIH6 and VIA4₁ binding activities of α -dystroglycan were all markedly increased after enzymatic deglycosylation. Other results suggest that α -dystroglycan oligosaccharides probably contain anionic sugars in addition to sialic acid and possibly rare α -linked GlcNAc moieties that could block complete enzymatic deglycosylation with currently available reagents.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated ConA (concanavalin A), ECA (*Erythrina cristagalli* agglutinin), WGA (wheat germ agglutinin) and ExtrAvidin were purchased from Sigma–Aldrich (St Louis, MO, U.S.A.). Peroxidase-conjugated LFA (*Limax flavus* agglutinin) and PNA (peanut agglutinin) were purchased from EY Laboratories (San Mateo, CA, U.S.A.). Biotin-conjugated GNL (*Galanthus nivalis*), GsII (*Griffonia simplicifolia* II) and *Hippocrepium* hybrid lectins were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). α -Dystroglycan-specific mAbs (monoclonal antibodies) IIH6 and VIA4₁ [10] were obtained from the University of Iowa Hybridoma Facility (IA, U.S.A.), and Upstate Biotechnology (Lake Placid, NY, U.S.A.) respectively. Affinity-purified chicken polyclonal antibodies to α -dystroglycan were prepared as described previously [17]. The DIG glycan detection kit and peroxidase-conjugated secondary antibodies were purchased from Roche Applied Science (Indianapolis, IN, U.S.A.). Purified mouse EHS laminin-1 was kindly provided by Dr H. Kleinman (Cell Biology Section, National Institute of Dental Research NIH, Bethesda, MD, U.S.A.).

Abbreviations used: ConA, concanavalin A; ECA, *Erythrina cristagalli* agglutinin; GsII, *Griffonia simplicifolia* II; LFA, *Limax flavus* agglutinin; mAb, monoclonal antibody; PNA, peanut agglutinin; WGA, wheat germ agglutinin.

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α -Dystroglycan purification

α -Dystroglycan was purified from rabbit skeletal muscle by the method of Brancaccio and co-workers [18] with several modifications. Freshly frozen rabbit back and leg muscle (300 g) was homogenized in 5 ml/g of 50 mM Tris/HCl (pH 7.4), 200 mM NaCl, 0.02 % NaN₃ and 0.2 mM PMSF, centrifuged for 20 min at 30000 g, and the resulting supernatant loaded on to a 5 cm $\times$ 14 cm DEAE-Sephacel column. After extensive washing, bound proteins were eluted in bulk with 230 ml of 50 mM Tris/HCl (pH 7.4), 500 mM NaCl and 0.02 % NaN₃. The DEAE eluate was circulated overnight at 4 °C through a 2.5 cm $\times$ 12 cm WGA-agarose column, which was washed extensively and bound proteins eluted in bulk with 100 ml of 0.3 M *N*-acetyl-D-glucosamine in 50 mM Tris/HCl (pH 7.4), 500 mM NaCl and 0.02 % NaN₃. The WGA eluate was diluted with 233 ml of 50 mM Tris/HCl (pH 7.4), 1.43 mM CaCl₂ and 1.43 mM MgCl₂, and circulated overnight through a 2.5 cm $\times$ 5 cm laminin-1-Sepharose column. After extensive washing with 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂, bound proteins were eluted in 5 ml fractions with 100 ml of 50 mM Tris/HCl (pH 7.4), 1 M NaCl and 10 mM EDTA. The fractions containing α -dystroglycan were identified by Western-blot analysis, pooled and further purified by CsCl gradient centrifugation as described previously [19]. Purified α -dystroglycan was exhaustively dialysed against water and quantified by A_{280} using $E_{280} = 0.83 \text{ cm}^2/\text{mg}$ [17]. Over ten trials, the method yielded on average 30 μg of purified α -dystroglycan starting with 300 g of skeletal muscle.

Enzyme treatments

Unless noted otherwise, 5 μg of purified rabbit skeletal α -dystroglycan was incubated with individual enzymes or various enzyme combinations for 16–20 h at 37 °C in 100 μl of 50 mM sodium phosphate at pH 5, pH 6 or pH 7. Enzyme digestions were terminated by the addition of 25 μl of 5 $\times$ Laemmli sample buffer (15 %, w/v, SDS, 0.575 M sucrose, 0.325 M Tris/HCl, pH 6.8 and 5 %, w/v, 2-mercaptoethanol), and 25 μl aliquots (containing 1 μg α -dystroglycan) were analysed by SDS/PAGE, lectin and Western blotting or laminin overlay as described below. The GLYCOPRO deglycosylation kit and PRO-LINK extender kit were purchased from Prozyme (San Leandro, CA, U.S.A.) and included the following enzymes with the concentrations used in parentheses: N-glycosidase F (50 units/ml), *Arthrobacter ureafaciens* sialidase (50 mU/ml), endo-O-glycosidase (12.5 mU/ml), β (1–4)galactosidase (30 mU/ml) and β -*N*-acetylglucosaminidase (0.4 unit/ml). The original source of enzymes provided in the GLYCOPRO deglycosylation kit and PRO-LINK extender kit used in the present study are no longer available directly from Prozyme but can be purchased in kits available from Calbiochem (San Diego, CA, U.S.A.), Sigma–Aldrich or QA-Bio (San Mateo, CA, U.S.A.).

Periodate oxidation

Purified skeletal muscle α -dystroglycan (5 μg) was incubated in 50 μl of 10 mM sodium metaperiodate, 50 mM sodium acetate (pH 5.5) for 0–6 h and the reaction terminated by the addition of 50 μl of 20 mM sodium disulphite and 25 μl of 5 $\times$ Laemmli sample buffer. Aliquots (25 μl) were analysed by SDS/PAGE, lectin and Western blotting or laminin overlay as described below.

SDS/PAGE, Western and lectin blotting

Samples were resolved on 3–12 % SDS/polyacrylamide gels and either stained with Coomassie Blue, Stains-All, or transferred on to nitrocellulose as described previously [11]. Western blot-

ting was performed as described previously [10]. Lectin blotting was performed by blocking nitrocellulose transfers with PBS containing 0.05 % Tween 20 followed by incubation with 1 $\mu\text{g}/\text{ml}$ of biotinylated or peroxidase-conjugated lectin in blocking buffer for 1 h at room temperature (25 °C). Blots incubated with biotinylated lectins were washed twice with blocking buffer and incubated for 1 h at room temperature with a 1:1000 dilution of peroxidase-conjugated ExtrAvidin in blocking buffer. Lectin blots were washed extensively with PBS before staining was developed in 20 mM Tris/HCl (pH 7.4) using 4-Cl-1-naphthol as substrate, or by enhanced chemiluminescence using Pierce SuperSignal Pico West substrate. Oxidation and digoxigenin labelling of blotted α -dystroglycan followed method B of the manufacturer's instructions provided with the DIG glycan detection kit. Images of gels, blots and chemiluminescence films were acquired using a UVP GelDoc imaging system and LabWorks image analysis software before importation into CorelDraw 10 for figure preparation.

Laminin-binding assays

Laminin-1 binding to blotted control and glycosidase-digested α -dystroglycan was assessed using a previously described laminin overlay assay [17]. Solid-phase radioligand and ELISA binding assays were performed as described previously [17,19]. Briefly, break-apart polystyrene microtitre wells coated overnight at 4 °C with 0.1 μg of purified laminin-1 or α -dystroglycan were blocked with 3 % (w/v) BSA in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂, and incubated with 1–80 nM ¹²⁵I- α -dystroglycan or 4 nM laminin-1 in blocking buffer. After two brief washes with blocking buffer, binding of ¹²⁵I- α -dystroglycan to immobilized laminin-1 was determined by breaking apart individual microtitre wells, which were counted in a Packard 5650 γ -counter [17]. The binding of unlabelled laminin-1 to immobilized α -dystroglycan was detected with affinity-purified rabbit polyclonal antibodies against laminin-1, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody and colour development with 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Colour development was quantified kinetically at 655 nm using a Molecular Dynamics Spectramax 340 UV-visible microplate reader.

RESULTS

Characterization of purified skeletal muscle α -dystroglycan

Although α -dystroglycan is typically purified from tissue homogenates or membrane-enriched fractions through various combinations of lectin, ion exchange and laminin-1 affinity chromatography, the method of Brancaccio and co-workers [18,20] is widely used because it is the least labour-intensive, requires only commercially available reagents, and can be applied to a variety of frozen tissues. The method involves a combination of DEAE-Sephacel and WGA-Sepharose chromatography of Tris-buffered saline extracts sometimes followed by laminin-1 affinity chromatography. When starting with rabbit skeletal muscle, we find that the method consistently yields fractions greatly enriched in α -dystroglycan immunoreactivity and laminin-binding activity. However, the presence of several residual glycoprotein contaminants potentially complicates the characterization of the carbohydrate structures present specifically on α -dystroglycan. We previously observed that α -dystroglycan sedimented to the densest regions of isopycnic CsCl gradients [19], presumably because the high negative charge density of α -dystroglycan [21,22] causes it to form a complex with heavy cesium ions.

Figure 1 Characterization of purified skeletal muscle α -dystroglycan

Shown in (A) are a Coomassie Blue-stained gel (CB) and an autoradiograph from a gel loaded with iodinated α -dystroglycan (^{125}I - α DG). Also shown are nitrocellulose transfers loaded with purified α -dystroglycan and blotted with polyclonal (α DG pAb) or mAb (IIH6) to α -dystroglycan, ConA, or laminin-1. Molecular mass standards ($\times 10^{-3}$) are indicated on the left. Shown in (B) is the amount of ^{125}I - α -dystroglycan specifically bound to microtitre wells coated with laminin-1 as a function of increasing α -dystroglycan concentration. Shown in (C) is the amount of laminin-1 bound to microtitre wells coated with purified α -dystroglycan in the presence of increasing concentrations of heparin or *N*-acetylneuraminic acid (NANA).

Therefore the α -dystroglycan-enriched fractions obtained after serial DEAE-, WGA- and laminin-1 chromatography were subjected to CsCl gradient centrifugation. The recovered protein appeared as a single broad band with an apparent M_r of 156 000 when stained with Coomassie Blue, radiolabelled on tyrosine residues with ^{125}I , or when transferred on to nitrocellulose and stained with polyclonal antibodies to α -dystroglycan core protein or mAb IIH6 to its oligosaccharides (Figure 1A). The 156 000 M_r protein also stained weakly with ConA (Figure 1A), *Galanthus nivalis* and *Hippeastrum* hybrid lectins (results not shown), which is consistent with the presence of high mannose *N*-linked oligosaccharides [23,24]. Blotted α -dystroglycan stained strongly when overlaid with purified laminin-1 (Figure 1A). When incubated at various concentrations in microtitre wells bearing immobilized laminin-1, the ^{125}I -labelled α -dystroglycan displayed saturable binding with a half-maximal concentration of 7 nM

Figure 2 Heterogeneous sialoglycosylation of skeletal muscle α -dystroglycan

Shown are identical nitrocellulose transfers loaded with equal amounts of purified α -dystroglycan incubated for 17 h at 37 °C in 50 mM sodium phosphate (pH 7.0) in the absence (Con), or presence of *A. ureafaciens*, *C. perfringens* or *V. cholerae* sialidase and stained with α -dystroglycan mAb IIH6, horseradish peroxidase-conjugated WGA or LFA.

(Figure 1B), which is in excellent agreement with the half-maximal concentration measured for ^{125}I -laminin-1 binding to immobilized skeletal muscle α -dystroglycan (8 nM) previously reported by Pall and co-workers [19]. Using an ELISA-based assay, we also confirmed that laminin-1 binding to purified skeletal muscle α -dystroglycan was inhibited by *N*-acetylneuraminic acid and heparin with measured IC_{50} values of 7 mM and 14 μM respectively (Figure 1C). α -Dystroglycan prepared by our modified method was also recently shown to bind recombinant laminin-2 in a calcium-dependent, but heparin-insensitive manner [25]. We conclude that the α -dystroglycan prepared with the additional CsCl gradient centrifugation step was devoid of any detectable glycoprotein contaminants and retained well-established laminin binding properties.

Heterogeneity in α -dystroglycan sialylation

We previously demonstrated that α -dystroglycan staining by WGA and *Maackia amurensis* agglutinin was completely ablated after digestion with *Vibrio cholerae* sialidase [10,11], but we later demonstrated that a subpopulation of skeletal muscle α -dystroglycan became reactive with the GalNAc-specific lectin *Vicia villosa* agglutinin after digestion with sialidase preparations from *Clostridium perfringens* or *Aureafaciens ureafaciens*, but not *V. cholerae* sialidase [17,26]. These results led us to conclude that skeletal muscle α -dystroglycan displays heterogeneity in its sialoglycosylation [17,26]. To further assess this possibility, we digested purified skeletal muscle α -dystroglycan with sialidases prepared from *A. ureafaciens*, *C. perfringens* or *V. cholerae* and performed blot overlays using peroxidase-conjugated WGA and LFA. WGA specifically binds *N*-acetylneuraminic acid, whereas LFA is more broadly reactive with both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid [27]. As previously reported [17], WGA binding to α -dystroglycan was completely abrogated after digestion with *A. ureafaciens*, *C. perfringens* or *V. cholerae* sialidase (Figure 2). In contrast, LFA binding to α -dystroglycan was lost after digestion with *A. ureafaciens*

or *C. perfringens* sialidases, but retained after digestion with *V. cholerae* sialidase (Figure 2). These results indicate that *V. cholerae* sialidase removes *N*-acetylneuraminic acid but not *N*-glycolylneuraminic acid moieties from α -dystroglycan and provides new evidence that skeletal muscle α -dystroglycan is heterogeneous with respect to its sialoglycosylation.

Enhanced laminin-1, mAb IIH6 and VIA4₁ binding to α -dystroglycan after enzymatic deglycosylation

We previously reported that enzymatic digestion with a combination of N-glycosidase F, *V. cholerae* sialidase and endo-O-glycosidase significantly altered the apparent M_r and lectin reactivity of skeletal muscle α -dystroglycan but had no effect on its laminin-1 binding activity [11]. Given the identification of SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man glycans on α -dystroglycan [14–16] and the results shown in Figure 2, it remained possible that our previous experiments [11] lacked the full complement of enzymes necessary to effect complete deglycosylation. Therefore we digested purified α -dystroglycan with a cocktail of five enzymes that together were expected to substantially degrade all *N*-linked and *O*-linked oligosaccharides known to modify skeletal muscle α -dystroglycan. The five enzymes were N-glycosidase F, *A. ureafaciens* sialidase, endo-O-glycosidase, β (1-4)galactosidase and β -*N*-acetylglucosaminidase, which are commercially available in kit form from several vendors (see the Materials and methods section). To simplify communication of the experiments, simultaneous digestion of α -dystroglycan with all five enzymes is operationally defined as 'GLYCOPRO' after the name of the commercial enzymatic deglycosylation kit used. Digestion with the GLYCOPRO kit effected an apparent M_r decrease of 40000 in skeletal muscle α -dystroglycan (Figure 3A, CB, α -DG pAb), which is the greatest shift that we have ever observed with any combination of glycosidases tested. However, GLYCOPRO-treated α -dystroglycan retained covalently bound carbohydrate based on digoxigenin-hydrazide incorporation after mild periodate oxidation (Figure 3A, Dig-Hz). Although GLYCOPRO digestion resulted in the complete loss of α -dystroglycan staining by sialic acid-reactive lectin LFA (Figure 3A), Stains-All binding to α -dystroglycan was only reduced 30 % (Figure 3A). Because Stains-All reacts with anionic constituents of the protein, carbohydrate and nucleic acid, these results suggest that sialic acid accounted for only one-third of the acidic constituents present on α -dystroglycan. α -Dystroglycan also became more strongly reactive with ConA after GLYCOPRO digestion (Figure 3A), possibly due to exposure of *O*-linked mannosyl residues within SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man glycans of α -dystroglycan.

Most surprisingly, GLYCOPRO-digested α -dystroglycan consistently exhibited markedly enhanced binding to laminin-1 and mAbs VIA4₁ and IIH6 (Figure 3B), all of which depend on the presence of unidentified oligosaccharides on α -dystroglycan [11]. The observed increase in α -dystroglycan binding to laminin-1, and mAbs VIA4₁ and IIH6 may be partly due to a more effective transfer of deglycosylated versus native α -dystroglycan because core protein-reactive polyclonal antibodies exhibited slightly increased reactivity for GLYCOPRO-digested α -dystroglycan (Figure 3A, α -DG pAb). However, the greatly enhanced laminin-1 binding activity after GLYCOPRO digestion strikingly conflicts with a previous study reporting the complete loss of laminin-1 binding to peripheral nerve α -dystroglycan after digestion with sialidase alone [24]. The results of Figure 3 suggest that digestion of α -dystroglycan with the five GLYCOPRO enzymes led to removal of oligosaccharides that normally repress laminin and carbohydrate-specific mAb binding.

Figure 3 Enzymatic deglycosylation of skeletal muscle α -dystroglycan

α -Dystroglycan was incubated for 20 h at 37 °C in 50 mM sodium phosphate, pH 7, in the absence (–) or presence (+) of all five enzymes provided in the GLYCOPRO (N-glycosidase F, *A. ureafaciens* sialidase, and endo-O-glycosidase) and PROLINK Extender [β (1-4)galactosidase and β -*N*-acetylglucosaminidase] enzymatic deglycosylation kits. In (A), equal amounts of control (–) and GLYCOPRO-treated (+) α -dystroglycan were resolved on SDS-polyacrylamide gels and stained with Coomassie Blue, or transferred on to nitrocellulose and stained with chicken polyclonal antibodies to α -dystroglycan core protein (α -DG pAb). Identical gels/transfers containing equal amounts of control and enzyme-treated α -dystroglycan were analysed for residual carbohydrate (Dig-Hz), Stains-All reactivity, or binding to the lectins LFA or ConA. The asterisk marks the 180000 M_r endo-O-glycosidase band present in the enzyme-treated samples. In (B), identically prepared transfers were analysed for laminin-1 binding (Ln-1 O/L), or for reactivity with mAbs IIH6 and VIA4₁.

Validation of glycosidase specificity

To more specifically test whether the GLYCOPRO enzyme cocktail was active on the known oligosaccharides of α -dystroglycan, we digested α -dystroglycan with different combinations of GLYCOPRO enzymes and performed blot overlays with a panel of lectins. *A. ureafaciens* sialidase digestion rendered α -dystroglycan strongly reactive with PNA, which was lost after digestion with endo-O-glycosidase but not by β (1-4)galactosidase and β -*N*-acetylglucosaminidase (Figure 4A). These results confirmed the presence and specific removal of sialylated core 1 oligosaccharides (Figure 4B) previously identified on skeletal muscle α -dystroglycan [10,28]. Based on its demonstrated modification with SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man glycans (Figure 4B), *A. ureafaciens* sialidase-digested α -dystroglycan was predicted to react with the lectin ECA, which specifically binds Gal β (1-4)GlcNAc but not SiaA α (2-3)Gal β (1-4)GlcNAc [29]. *A. ureafaciens* sialidase digestion rendered α -dystroglycan strongly reactive with ECA (Figure 4A), confirming the exposure of Gal β (1-4)GlcNAc glycans. ECA staining was completely ablated by digestion with β (1-4)galactosidase and glucosaminidase but was unaffected by endo-O-glycosidase (Figure 4A). Interestingly, simultaneous digestion with *A. ureafaciens* sialidase, β (1-4)galactosidase and β -*N*-acetylglucosaminidase made α -dystroglycan strongly reactive with GsII lectin (Figure 4A), which is specific for terminal α - and β -linked GlcNAc [27]. We further observed that digestion of α -dystroglycan with *A. ureafaciens* sialidase alone increased ConA reactivity, which was more

Figure 4 Skeletal muscle α -dystroglycan contains latent glyco-epitopes reactive with conA and GsII

Shown in (A) are identical nitrocellulose transfers loaded with equal amounts of purified α -dystroglycan incubated for 18 h at 37 °C in 50 mM sodium phosphate (pH 7) in the absence or presence of the indicated enzymes and stained with α -dystroglycan mAb IIH6, PNA, ECA, GsII lectin or ConA. Shown in (B) are the sialylated core 1 (top) and *O*-mannosyl (bottom) oligosaccharide structures known to modify α -dystroglycan with predicted effect of each glycosidase on oligosaccharide structure and lectin reactivity.

dramatically enhanced by digestion with β (1-4)galactosidase and glucosaminidase (Figure 4A). Taken together, the experiments depicted in Figures 3 and 4 demonstrate that digestion of α -dystroglycan with the GLYCOPRO regime substantially degraded the terminal dissaccharide of the sialyl *O*-mannosyl glycan thought to be important for laminin binding, yet α -dystroglycan binding to laminin-1 and mAbs IIH6 and VIA4₁ was paradoxically enhanced.

To assess whether other known carbohydrate modifications may block enzymatic removal of the established *O*-linked core structures present on α -dystroglycan, we evaluated the effect of 11 additional enzymes on the laminin-1, antibody and lectin binding activities of α -dystroglycan either alone or in combination with the five GLYCOPRO enzymes (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/390/bj3900303add.htm>). The enzymes targeted α -linked Gal and GalNAc, α -linked fucose, β -linked glucuronic acid, α - and β -linked Man, the naturally occurring sialic acid analogue 2-keto-3-deoxy-D-glycero-D-galactononic acid as well as sulphation and phosphorylation. However, none of the enzymes tested had any effect on the laminin-1, antibody or lectin binding activities of α -dystroglycan.

Evidence for modification of α -dystroglycan by α -linked GlcNAc

Gastric mucins are uniquely modified by ConA-reactive glyco-epitopes that resist, or are even enhanced by periodate oxidation [30]. This so-called paradoxical ConA staining has subsequently been shown to be due to terminal α -linked GlcNAc [31,32], a carbohydrate linkage recognized by both ConA and GsII [27] and for which no specific exoglycosidase is currently available. Since skeletal muscle α -dystroglycan exhibited latent reactivity with both ConA and GsII that was resistant to digestion with a wide

Figure 5 Periodate oxidation of α -dystroglycan

Shown are identical nitrocellulose transfers loaded with equal amounts of skeletal muscle α -dystroglycan incubated at room temperature in 10 mM sodium metaperiodate for the indicated times and analysed for laminin-1 binding (Ln-1 O/L), or for reactivity with chicken polyclonal antibodies to α -dystroglycan (α -DG pAb), mAb IIH6 or ConA.

variety of enzymes (Figures 3 and 4, Supplementary Table 1), we examined the effect of periodate oxidation on α -dystroglycan ConA reactivity (Figure 5). We observed that prolonged (2–16 h) exposure of α -dystroglycan to 10 mM periodate at room temperature caused a shift in apparent M_r from 156 000 to 59 000 with complete abrogation of IIH6 and laminin-1 binding activity (Figure 5A). We also observed that prolonged periodate oxidation resulted in the complete loss of Stains-All binding to α -dystroglycan (Figure 5B), which suggests that anionic carbohydrate is required for Stains-All binding to α -dystroglycan. After 30 min of periodate oxidation, we detected an apparent intermediate of 88 000 M_r that retained strong IIH6 and laminin-1 binding activity. Most interestingly, ConA reactivity was retained in both the 59 000 and 88 000 M_r species up to 60 min of periodate oxidation, reminiscent of the time course for paradoxical ConA staining in gastric mucin [31].

DISCUSSION

The presence of two novel *O*-mannosyl glycan structures on α -dystroglycan has been well documented using established analytical methods [14–16]. *O*-Mannosyl oligosaccharides of unknown structure are also clearly important for α -dystroglycan function *in vivo* because mutations in genes encoding demonstrated or putative glycosyltransferases are associated with hypoglycosylated α -dystroglycan, loss of its extracellular ligand binding activities and muscular dystrophy [3,6]. However, the specific importance of Sia α 2-3Gal β 1-4GlcNAc β 1-2Man in the extracellular ligand binding activity of α -dystroglycan rests tenuously on two dubious experiments. In the first experiment, sialic acid [24] and α 2-3 sialyl *N*-acetylglucosamine [14] were shown to inhibit laminin binding to peripheral nerve α -dystroglycan with IC₅₀ values of approx. 6 mM and > 10 mM respectively. Using a similar assay, we confirmed that sialic acid inhibited laminin binding to α -dystroglycan with a similar IC₅₀ value of 7 mM. However, the potential significance of this result is tempered when compared directly with heparin (Figure 1), which inhibited binding with a 500-fold lower IC₅₀. It was also reported that digestion of α -dystroglycan with sialidase resulted in the complete loss of laminin binding activity [24]. Unfortunately, this second experiment lacked controls proving that the loss of laminin binding was not due to α -dystroglycan core protein degradation

caused by a protease contaminant within the sialidase preparation. Moreover, neither we [11,17] nor several other groups [12,22,33] observe a loss of extracellular matrix ligand binding activity when α -dystroglycan from several different tissues was exhaustively digested with a wide variety of sialidase preparations.

In the present study, we observed substantial retention of laminin binding activity in an 88000 M_r α -dystroglycan intermediate detected after incubation with 10 mM periodate for 30 min at room temperature (Figure 5). In contrast, sialic acid is readily oxidized within 20 min by 1 mM periodate at 0°C. Most importantly, we enzymatically removed all detectable sialic acid and galactose moieties from SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man glycans on α -dystroglycan, but observed increased laminin-1, IIH6 and VIA4₁ binding activities (Figures 3 and 4). Finally, Matsumura and co-workers [34] recently identified a distal myopathy patient with mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase gene. Although this patient exhibited gross hyposialylation of skeletal muscle glycoproteins, the laminin binding activity of α -dystroglycan was not impaired. Taken together, these results suggest that the terminal SiaA α 2-3Gal moieties on *O*-mannosyl glycans of skeletal muscle α -dystroglycan are not essential determinants for laminin binding activity and that their removal leads to enhanced binding activity for laminin-1, and carbohydrate-specific mAbs IIH6 and VIA4₁.

Our results further indicate that α -dystroglycan is modified with anionic sugars distinct from sialic acid because its Stains-All reactivity was only reduced by 30% after sialidase digestion (Figure 3) but completely abolished after periodate oxidation (Figure 5). Consistent with our results, sialidase-resistant acidic oligosaccharides were previously isolated from peripheral nerve α -dystroglycan, but have not been further characterized (see Figure 6 of [14]). These anionic sugars may also provide key acidic functional groups necessary for calcium co-ordinated binding of α -dystroglycan to laminin [35]. Finally, our results suggest that oligosaccharides on α -dystroglycan may contain α -linked GlcNAc substituents, which could explain its resistance to complete enzymatic deglycosylation. Digestion of α -dystroglycan with sialidase and β (1-4)galactosidase revealed a very robust enhancement of ConA staining and latent GsII reactivity (Figure 4B). Certainly, a portion of GsII reactivity in sialidase and β (1-4)galactosidase digested α -dystroglycan may be due to undigested GlcNAc β 1-2Man cores within SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man glycans. However, the strong ConA and GsII staining remaining after GLYCOPRO digestion could not be eliminated by a wide array of enzymes tested (Supplementary Table 1). To our knowledge, the only carbohydrate structure recognized by both ConA and GsII is terminal α -linked GlcNAc [27]. Furthermore, the ConA reactivity of α -dystroglycan was also observed to withstand prolonged periodate oxidation (Figure 5), which is a notable feature of the α -linked GlcNAc terminated 'paradoxical' ConA epitope so far only detected on gastric mucins [30–32]. Interestingly, mutations in the LARGE gene lead to hypoglycosylated α -dystroglycan, loss of its extracellular ligand binding activities and muscular dystrophy [3,6], whereas overexpression of LARGE rescued these defects [8]. Although the biochemical function of the LARGE gene product is not certain, sequence analysis identified two domains homologous with bacterial α -glycosyltransferase and mammalian *N*-acetylglucosaminyltransferase respectively [36,37]. Unfortunately, currently there exists no purified glycosidase that can specifically remove α -linked GlcNAc [31,32] so its confirmation in α -dystroglycan awaits further investigation using biosynthetic approaches [7–9] and/or MS. With respect to the latter method, our highly purified and extensively characterized preparation of

skeletal muscle α -dystroglycan will provide an ideal substrate for analysis.

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REFERENCES

- Blake, D. J., Weir, A., Newey, S. E. and Davies, K. E. (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol. Rev.* **82**, 291–329
- Ervasti, J. M. (2003) Costameres: the Achilles' heel of Herculean muscle. *J. Biol. Chem.* **278**, 13591–13594
- Michele, D. E. and Campbell, K. P. (2003) Dystrophin–glycoprotein complex: post-translational processing and dystroglycan function. *J. Biol. Chem.* **278**, 15457–15460
- Cote, P. D., Moukhles, H., Lindenbaum, M. and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat. Genet.* **23**, 338–342
- Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R. et al. (2002) Disruption of DAG1 in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. *Cell (Cambridge, Mass.)* **110**, 639–648
- Muntoni, F., Brockington, M., Torelli, S. and Brown, S. C. (2004) Defective glycosylation in congenital muscular dystrophies. *Curr. Opin. Neurol.* **17**, 205–209
- Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U. and Endo, T. (2004) Demonstration of mammalian protein *O*-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 500–505
- Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P. et al. (2004) LARGE can functionally bypass α -dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nat. Med.* **10**, 696–703
- Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B. et al. (2004) Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell (Cambridge, Mass.)* **117**, 953–964
- Ervasti, J. M. and Campbell, K. P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell (Cambridge, Mass.)* **66**, 1121–1131
- Ervasti, J. M. and Campbell, K. P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122**, 809–823
- Bowe, M. A., Deyst, K. A., Leszyk, J. D. and Fallon, J. R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. *Neuron* **12**, 1173–1180
- Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K. and Sudhof, T. C. (2001) A stoichiometric complex of neuroligins and dystroglycan in brain. *J. Cell Biol.* **154**, 435–445
- Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A. and Endo, T. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α -dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α -dystroglycan with laminin. *J. Biol. Chem.* **272**, 2156–2162
- Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A. and Endo, T. (1998) Detection of *O*-mannosyl glycans in rabbit skeletal muscle α -dystroglycan. *Biochim. Biophys. Acta* **1425**, 599–606
- Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R. and Dell, A. (1998) Structural analysis of sequences *O*-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified from sheep brain. *J. Biol. Chem.* **273**, 23698–23703
- Ervasti, J. M., Burwell, A. L. and Geissler, A. L. (1997) Tissue-specific heterogeneity in α -dystroglycan sialoglycosylation: skeletal muscle α -dystroglycan is a latent receptor for *Vicia villosa* agglutinin B₄ masked by sialic acid modification. *J. Biol. Chem.* **272**, 22315–22321
- Brancaccio, A., Schulthess, T., Gesemann, M. and Engel, J. (1995) Electron microscopic evidence for a mucin-like region in chick muscle α -dystroglycan. *FEBS Lett.* **368**, 139–142
- Pall, E. A., Bolton, K. M. and Ervasti, J. M. (1996) Differential heparin inhibition of skeletal muscle α -dystroglycan binding to laminins. *J. Biol. Chem.* **271**, 3817–3821
- Gesemann, M., Brancaccio, A., Schumacher, B. and Ruegg, M. A. (1998) Agrin is a high-affinity binding protein of dystroglycan in non-muscle tissue. *J. Biol. Chem.* **273**, 600–605

- 21 Gee, S. H., Blacher, R. W., Douville, P. J., Provost, P. R., Yurchenco, P. D. and Carbonetto, S. (1993) Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. *J. Biol. Chem.* **268**, 14972–14980
- 22 Yamamoto, H., Hagiwara, Y., Mizuno, Y., Yoshida, M. and Ozawa, E. (1993) Heterogeneity of dystrophin-associated proteins. *J. Biochem. (Tokyo)* **114**, 132–139
- 23 Smalheiser, N. R. and Kim, E. (1995) Purification of cranin, a laminin binding membrane protein: identity with dystroglycan and reassessment of its carbohydrate moieties. *J. Biol. Chem.* **270**, 15425–15433
- 24 Yamada, H., Chiba, A., Endo, T., Kobata, A., Anderson, V. B., Hori, H., Fukuta-Ohi, H., Kanazawa, I., Campbell, K. P., Shimizu, T. et al. (1996) Characterization of dystroglycan–laminin interaction in peripheral nerve. *J. Neurochem.* **66**, 1518–1524
- 25 Smirnov, S. P., McDearmon, E. L., Li, S., Ervasti, J. M., Tryggvason, K. and Yurchenco, P. D. (2002) Contributions of the LG modules and furin processing to laminin-2 functions. *J. Biol. Chem.* **277**, 18928–18937
- 26 McDearmon, E. L., Combs, A. C. and Ervasti, J. M. (2001) Differential *Vicia villosa* agglutinin reactivity identifies three distinct dystroglycan complexes in skeletal muscle. *J. Biol. Chem.* **276**, 35078–35086
- 27 Goldstein, I. J. and Poretz, R. D. (1986) Isolation, Physicochemical Characterization, and Carbohydrate-Binding Specificity of Lectins, Academic Press, Orlando
- 28 McDearmon, E. L., Combs, A. C. and Ervasti, J. M. (2003) Core 1 glycans on alpha-dystroglycan mediate laminin-induced acetylcholine receptor clustering but not laminin binding. *J. Biol. Chem.* **278**, 44868–44873
- 29 Teneberg, S., Angstrom, J., Jovall, P. A. and Karlsson, K. A. (1994) Characterization of binding of Gal beta 4GlcNAc-specific lectins from *Erythrina cristagalli* and *Erythrina corallodendron* to glycosphingolipids. Detection, isolation, and characterization of a novel glycosphingolipid of bovine buttermilk. *J. Biol. Chem.* **269**, 8554–8563
- 30 Katsuyama, T. and Spicer, S. S. (1978) Histochemical differentiation of complex carbohydrates with variants of the concanavalin A-horseradish peroxidase method. *J. Histochem. Cytochem.* **26**, 233–250
- 31 Hotta, K., Goso, K. and Kato, Y. (1982) Human gastric glycoproteins corresponding to paradoxical concanavalin A staining. *Histochemistry* **76**, 107–112
- 32 Ishihara, K., Kurihara, M., Goso, Y., Urata, T., Ota, H., Katsuyama, T. and Hotta, K. (1996) Peripheral α -linked *N*-acetylglucosamine on the carbohydrate moiety of mucin derived from mammalian gastric gland mucous cells: epitope recognized by a newly characterized monoclonal antibody. *Biochem. J.* **318**, 409–416
- 33 Smalheiser, N. R. (1993) Cranin interacts specifically with the sulfatide-binding domain of laminin. *J. Neurosci. Res.* **36**, 528–538
- 34 Saito, F., Tomimitsu, H., Arai, K., Nakai, S., Kanda, T., Shimizu, T., Mizusawa, H. and Matsumura, K. (2004) A Japanese patient with distal myopathy with rimmed vacuoles: missense mutations in the epimerase domain of the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) gene accompanied by hyposialylation of skeletal muscle glycoproteins. *Neuromuscul. Disord.* **14**, 158–161
- 35 Hohenester, E., Tisi, D., Talts, J. F. and Timpl, R. (1999) The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin. *Mol. Cell* **4**, 783–792
- 36 Grewal, P. K., Holzfeind, P. J., Bittner, R. E. and Hewitt, J. E. (2001) Mutant glycosyltransferase and altered glycosylation of α -dystroglycan in the myodystrophy mouse. *Nat. Genet.* **28**, 151–154
- 37 Peyrard, M., Seroussi, E., Sandberg-Nordqvist, A. C., Xie, Y. G., Han, F. Y., Fransson, I., Collins, J., Dunham, I., Kost-Alimova, M., Imreh, S. et al. (1999) The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 598–603

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