

**HNK-1 sulfotransferase-dependent sulfation regulating laminin-binding glycans
occurs in the post-phosphoryl moiety on α -dystroglycan**

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Running title: HNK-1ST-dependent post-phosphoryl modification on α -DG

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Keywords: α -dystroglycan/HNK-1ST/laminin-binding glycan/LARGE/sulfation

Abstract

Dystroglycan (DG) is a cell surface glycoprotein that connects extracellular matrix molecules to the intracellular cytoskeleton, functioning as mechanical and signaling axes in various physiological events. Since the ligand binding activity of DG strictly depends on *O*-mannosyl glycans attached to its extracellular α -DG subunit, aberrant glycosylation causes dystroglycanopathy, a subclass of congenital muscular dystrophy. Accumulating evidence shows that like-acetylglucosaminyltransferase (LARGE), a glycosyltransferase involved in the biosynthesis of a phosphodiester-linked modification on *O*-mannose, is essential for α -DG to gain the ligand binding activity. We previously reported that human natural killer-1 sulfotransferase (HNK-1ST), which was originally reported as one of the enzymes responsible for HNK-1 glyco-epitope, had an ability to suppress the glycosylation and function of α -DG. In this study, we investigated how HNK-1ST regulates the glycosylation of α -DG using deletion and mutation analyses. We generated an α -DG mutant which has only one threonine residue capable of being modified by LARGE. Focusing on the single post-phosphoryl modification site, we found that HNK-1ST showed an almost complete inhibition of the LARGE-dependent modification and transferred a sulfate group to the phosphodiester-linked moiety on *O*-mannose. Furthermore, using an in vitro enzymatic assay system, we demonstrated that the sulfated α -DG by HNK-1ST is no longer glycosylated by LARGE. These results illustrate one possible glycosylation pathway where α -DG function is regulated by opposing actions of HNK-1ST and LARGE.

Introduction

At the cell-extracellular matrix (ECM) interface, molecular interactions are successfully conducted by various glycosylated molecules tethered on the plasma membrane, including glycoproteins and glycolipids (Ohtsubo and Marth 2006). Among them, dystroglycan (DG), a membrane-spanning glycoprotein, is present in various tissues and functions as a receptor for several ECM components such as laminin, agrin, and perlecan (Ibraghimov-Beskrovnaya et al. 1992; Barresi and Campbell 2006). DG undergoes various posttranslational processing to gain its functionally mature form during the secretory pathway from the endoplasmic reticulum (ER) to the plasma membrane. After the translation, DG polypeptide is cleaved into two subunits, α - and β -DG (Holt et al. 2000). α -DG consists of three domains, N-terminal and C-terminal globular domains and a central mucin-like domain, while β -DG has a transmembrane domain (Barresi and Campbell 2006). These two subunits remain noncovalently associated, and thereby the ligand binding unit α -DG is anchored on the plasma membrane by β -DG, which in turn couples to the cytoskeletal molecules, together organizing physical linkages between the cell and ECM (Ervasti and Campbell 1993). Passing through the ER and Golgi apparatus, extensive glycosylation occurs in the α -subunit of DG, which is the most functionally important processing (Michele and Campbell 2003; Barresi and Campbell 2006). In particular, *O*-mannosyl modification on the mucin-like domain of α -DG plays an essential role in the α -DG-ligand interaction (Michele and Campbell 2003; Barresi and Campbell 2006). Following the glycosylation process, the N-terminal domain of α -DG is cleaved off by furin, a Golgi-resident proprotein convertase. The N-terminal domain is required for the proper glycosylation of α -DG as a recognition scaffold for the glycosyltransferase (Kanagawa et al. 2004), but has little effect on the interaction of glycans on α -DG with its ligands (Singh et al. 2004).

Since the *O*-mannosyl modification on α -DG is crucial for the binding to its ligands, disruption of α -DG glycosylation causes the loss of function and underlies the molecular pathogenesis of several types of congenital muscular dystrophy associated with brain and eye abnormalities, classified as dystroglycanopathy (Michele et al. 2002; Muntoni et al. 2008). While the precise glycan structure serving as a laminin-binding epitope has not yet been

determined, two rare types of modification attached to *O*-linked mannose on α -DG have been found to date. An *O*-mannosyl tetrasaccharide (Sia α 2-3Gal β 1-4GlcNAc β 1-2Man) was discovered in bovine peripheral nerve α -dystroglycan (Chiba et al. 1997) and was demonstrated to be synthesized by protein *O*-mannosyltransferase 1 (POMT1), POMT2, and protein *O*-mannose β -1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) (Yoshida et al. 2001; Manya et al. 2004). The mutations in the genes encoding POMT1/2 and POMGnT1 cause muscular dystrophies with abnormal α -DG glycosylation designated as Walker-Warburg syndrome and muscle-eye-brain disease, respectively (Yoshida et al. 2001; Beltran-Valero de Bernabe et al. 2002; van Reeuwijk et al. 2005), implicating the importance of the *O*-mannosyl tetrasaccharide in α -DG function. Another *O*-mannosyl modification essential for α -DG function is a phosphodiester-linked structure present at the C-6 hydroxyl group of *O*-mannose (Yoshida-Moriguchi et al. 2010). Although the enzyme responsible for the phosphorylation of mannose is unknown at present, three known or putative glycosyltransferases, fukutin, fukutin-related protein (FKRP), and like-acetylglucosaminyltransferase (LARGE), have been shown to be involved in the assembly of the post-phosphoryl modification (Yoshida-Moriguchi et al. 2010; Kuga et al. 2012). The mutations in each gene encoding fukutin, FKRP, and LARGE are associated with the pathogenesis of muscular dystrophy (Kobayashi et al. 1998; Brockington et al. 2001; Longman et al. 2003). It has been recently demonstrated that LARGE is a bifunctional glycosyltransferase with glucuronyl- and xylosyltransferase activities, synthesizing disaccharide repeats of glucuronic acid (GlcA) and xylose (Xyl) (Inamori et al. 2012). Furthermore, the post-phosphoryl modification is absent in the myodystrophy mouse (*Large*^{myd}) harboring a spontaneous mutation in *LARGE* (Yoshida-Moriguchi et al. 2010; Inamori et al. 2012). While the precise functions of fukutin and FKRP including their enzymatic activities remain unclear, their dysfunctions also lead to the disappearance of the post-phosphoryl modification (Beedle et al. 2012; Kuga et al. 2012).

We previously found that human natural killer-1 sulfotransferase (HNK-1ST) could suppress the glycosylation and function of α -DG by transferring sulfate groups to α -DG (Nakagawa et al. 2012). HNK-1ST was originally reported as a sulfotransferase involved in the

biosynthesis of HNK-1 carbohydrate, a nervous system-specific glyco-epitope attached to the non-reducing end of glycans (Bakker et al. 1997; Ong et al. 1998). HNK-1 carbohydrate displays spatiotemporally regulated expression patterns and is carried by limited kinds of glycoproteins such as immunoglobulin-superfamily adhesion molecules (e.g. NCAM, L1, and P0) and several extracellular proteins (e.g. tenascin-C and phosphacan) or glycolipids (Kizuka and Oka 2012). In general, HNK-1ST acts in combination with one of two responsible glucuronyltransferases (GlcAT-P or GlcAT-S) to synthesize HNK-1 carbohydrate and contributes to the regulation of neural functions (Senn et al. 2002; Yamamoto et al. 2002; Kizuka et al. 2006; Morita et al. 2009). In contrast, the α -DG-regulating function of HNK-1ST does not require either glucuronyltransferase and is independent of HNK-1 biosynthesis (Nakagawa et al. 2012). However, further information about its precise mode of action, including the specific site of α -DG at which the sulfate group is modified, has yet to be provided.

In the present study, using deletion and mutation analyses, we investigated the mechanism of HNK-1ST-mediated suppression of α -DG glycosylation. Analyzing an α -DG mutant which has a single modification site for LARGE, we found that HNK-1ST almost completely inhibited the LARGE-dependent modification by transferring a sulfate group to the phosphodiester-linked structure on *O*-mannose. Furthermore, we showed that α -DG pre-sulfated by HNK-1ST was no longer employed as an acceptor substrate by LARGE using an in vitro enzyme assay system. Our findings shed light on the molecular mechanisms underlying the functional diversity of HNK-1ST as well as the elaborately regulated glycosylation of α -DG.

Results

Deletion and mutation analyses reveal the characteristic expression of laminin-binding glycans at distinct sites on α -DG

Since laminin-binding glycans are expressed on the central region of α -DG, called the mucin-like domain (Gln313 to Ser485 in human α -DG) (Barresi and Campbell 2006), we speculated that HNK-1ST transfers sulfate groups to this domain. To determine the specific sites on α -DG where HNK-1ST-dependent sulfation takes place, we employed a deletion analysis of the mucin-like domain of α -DG. Using a construct encoding α -DG fused to a human IgG Fc fragment (α -DG-Fc), we generated two deletion mutants by utilizing restriction sites in α -DG and termed them Δ mucin1-Fc and Δ mucin2-Fc, which lack Arg311 through Ile370 and Val368 through Arg461, respectively (Figure 1A). These constructs were transfected in CHO-K1 cells, and the expressed proteins were collected from culture medium by protein G Sepharose-beads and analyzed by Western blotting. The immunoblot with anti-Fc antibody showed that the two deletion mutants were properly expressed (Figure 1B). Compared to the full length α -DG-Fc, the molecular weight of Δ mucin2-Fc was decreased due to the deleted amino acids, while that of Δ mucin1-Fc was increased due to the presence of N-terminal domain resulting from the disruption of the recognition motif RXRR (Arg309 to Arg312) for the proprotein convertase furin (Kanagawa et al. 2004; Singh et al. 2004). We then assessed the ligand binding activity and glycosylation state of these mutants by the laminin overlay assay and immunoblotting with IIH6 monoclonal antibody, which recognizes the functionally glycosylated form of α -DG (Ervasti and Campbell 1993). When expressed alone, α -DG-Fc and Δ mucin2-Fc but not Δ mucin1-Fc exhibited laminin-binding activity, but these laminin-binding moieties were hardly detected by IIH6 antibody (Figure 1B). In contrast, co-expression with myc- and His-tagged LARGE (LARGE-myc) resulted in the production of the high-molecular laminin-binding form that could react with IIH6 antibody not only in α -DG-Fc and Δ mucin2-Fc but also in Δ mucin1-Fc (Figure 1B). To determine whether or not the laminin-binding activity produced on Δ mucin1-Fc by LARGE overexpression was derived from the post-phosphoryl modification, we employed cold aqueous hydrofluoric acid (HFaq) treatment, which specifically hydrolyzes phosphoester linkages (Yoshida-Moriguchi et al. 2010; Zhang et al. 2012). As a

result, the LARGE-induced laminin-binding activity of Δ mucin1-Fc almost disappeared in the HFAQ-treated membrane as in the case of α -DG-Fc and Δ mucin2-Fc (Supplementary data, Figure S1A). Regarding the reaction specificity of HFAQ treatment, we confirmed that other glyco-epitope, HNK-1, did not disappear by this treatment (Supplementary data, Figure S1B and C). These results suggest that Δ mucin1-Fc has at least one potential post-phosphoryl glycosylation site, which LARGE could act on. Similarly, the laminin-binding activities detected on the solely expressed α -DG-Fc and Δ mucin2-Fc disappeared by HFAQ treatment (Figure 1C), indicating that α -DG-Fc and Δ mucin2-Fc are modified by the post-phosphoryl modification in CHO-K1 cells, presumably due to the action of endogenous LARGE. Therefore, our findings that the laminin-binding activity was not detected in Δ mucin1-Fc without LARGE overexpression although Δ mucin1-Fc had potential site(s) for LARGE, suggest a small number of LARGE-dependent modification sites in Δ mucin1-Fc.

Previous studies have shown that the first half (Gln313 to Pro408) of the mucin-like domain is sufficient for the ligand binding activity of α -DG and that the second half (Gly409 to Ser485) does not undergo the modification of laminin-binding glycans even in LARGE-overexpressing cells (Kanagawa et al. 2004). Thus, considering that Δ mucin1-Fc was glycosylated by LARGE, the region between Arg371 and Pro408 is predicted to contain at least one site for the LARGE-dependent glycosylation. While the amino acid sequence from Arg371 to Pro408 contains nine potential *O*-glycosylation sites including eight Thr and one Ser residues, the specific threonine, Thr379, is reported to bear 6-phosphorylated *O*-mannose that could be further modified by LARGE (Yoshida-Moriguchi et al. 2010). Therefore, to confirm whether Thr379 is modified by LARGE, we generated α -DG constructs that harbored a mutation of Thr379 to Ala using α -DG-Fc and Δ mucin1-Fc as templates and termed them α -DG-T379A and Δ m1-T379A, respectively (Figure 2A). When expressed with LARGE-myc in CHO-K1 cells, Δ m1-T379A was found to be hardly modified by IIH6-positive laminin-binding glycans (Figure 2B). In contrast, T379A mutation on the wild-type α -DG-Fc (α -DG-T379A) caused only slight reductions in laminin-binding and IIH6 immunoreactivity (Figure 2B). These results showed that Thr379 was indeed glycosylated by LARGE and was a major modification site within the mucin-like domain of Δ mucin1-Fc (Arg371 to Ser485).

HNK-1ST almost entirely inhibits the laminin-binding glycan synthesis by LARGE

We recently revealed that HNK-1ST could counteract the effect of LARGE, resulting in the reduction of α -DG function (Nakagawa et al. 2012). However, we could not propose mechanistic insights about the α -DG-modulating effect of HNK-1ST including the specific site where the sulfate group is attached. To address this issue, we utilized Δ mucin1-Fc to investigate the functional interaction between HNK-1ST and LARGE since it has only one major LARGE modification site. In the presence of HNK-1ST-EGFP, the IIH6-positive laminin-binding glycans generated by LARGE on Δ mucin1-Fc as well as the full length α -DG-Fc were markedly suppressed (Figure 3). However, the apparent inhibitory effect of HNK-1ST on the full length α -DG-Fc was somewhat mild considering its residual laminin-binding, whereas that on Δ mucin1-Fc was much more drastic because of the almost complete disappearance of its laminin-binding (Figure 3). This difference in degree of the suppressive effect of HNK-1ST was presumably due to the numbers of potential modification sites present in α -DG-Fc and Δ mucin1-Fc. The result that HNK-1ST showed almost complete inhibition of the LARGE-dependent glycosylation on Δ mucin1-Fc, which had a single LARGE modification site, suggests that HNK-1ST functions in the same glycosylation pathway as LARGE.

HNK-1ST transfers a sulfate group downstream of the phosphodiester linkage of O-mannose

To examine whether HNK-1ST transfers a sulfate group to the post-phosphoryl modification site upon which LARGE acts, resulting in the direct inhibition of the laminin-binding glycan attachment, we carried out a radiolabeled ^{35}S -sulfate group incorporation assay. As a result, incorporation of ^{35}S -sulfate groups was detected on Δ mucin1-Fc in the presence of HNK-1ST-EGFP (Figure 4A). By contrast, Δ m1-T379A co-expressed with HNK-1ST-EGFP showed a remarkably decreased ^{35}S signal compared to Δ mucin1-Fc, demonstrating that HNK-1ST mainly transfers a sulfate group onto the glycan modified on Thr379 (Figure 4A). A faint ^{35}S band remained in Δ m1-T379A suggests the presence of other weak potential site(s) for HNK-1ST presumably located within *O*-glycosylation sites between Arg371 and Pro408 mentioned above. Next, to further investigate where the sulfate group is attached, we treated the

radiolabeled Δ mucin1-Fc with cold HFAQ. HNK-1ST-dependent ^{35}S signal on Δ mucin1-Fc almost disappeared by HFAQ treatment, indicating that HNK-1ST-dependent sulfation site is present in the phosphodiester-linked moiety attached on *O*-mannose (Figure 4B). As shown in Figure S1A and Figure 2B, LARGE generates a laminin-binding glycan via the phosphodiester linkage at Thr379. Taken together, these findings indicate that both HNK-1ST and LARGE are involved in the post-phosphoryl modification on the specific threonine residue but they have opposite effects on the functional maturation of α -DG.

Pre-sulfated α -DG no longer undergoes glycosylation by LARGE in vitro

To investigate whether the sulfate modification on α -DG is sufficient for impeding the enzymatic reaction of LARGE, we employed an in vitro assay system to assess whether the sulfated α -DG is utilized as a substrate for LARGE. The assay system for LARGE enzymatic activity, the polymerization of GlcA and Xyl, was constructed according to a recently established method (Inamori et al. 2012), with the full length LARGE-myc proteins used as enzyme sources instead of a secreted form of LARGE. As acceptor substrates, sulfated or non-sulfated Δ mucin1-Fc proteins were prepared from Δ mucin1-Fc expressing cells with or without co-transfection of HNK-1ST-EGFP. The polymerization reactions of LARGE toward these substrates were then assessed by incubation with LARGE-myc protein, which had been independently prepared from LARGE-myc expressing cells using Ni-NTA conjugated beads. The reaction products were subjected to Western blotting to evaluate whether the Δ mucin1-Fc substrates were modified by laminin-binding glycans. Using the non-sulfated Δ mucin1-Fc as a substrate (Δ mucin1-Fc), laminin-binding glycans were observed only in the presence of the donor substrates, UDP-GlcA and UDP-Xyl, indicating that LARGE generates the repeating unit of GlcA and Xyl on Δ mucin1-Fc, which confers laminin-binding activity (Figure 5). However, in the case of the sulfated Δ mucin1-Fc (Δ m1+HNK-1ST), laminin-binding glycans were hardly detected on the reaction products, as was the case for Δ m1-T379A that lacked the LARGE modification site (Figure 5). These results suggest that the sulfate group on α -DG transferred by HNK-1ST interrupts the sugar polymerization by LARGE probably due to the disruption of the acceptor structure required for the LARGE enzymatic reaction.

Discussion

Accumulating evidence indicates that the post-phosphoryl modification extended from *O*-linked mannose on α -DG plays a pivotal role in the α -DG-ligand interaction (Yoshida-Moriguchi et al. 2010; Beedle et al. 2012; Inamori et al. 2012; Kuga et al. 2012). In agreement with this notion, the post-phosphoryl modification is lacking and the free phosphate group is exposed on the *O*-linked mannose in the tissue preparation from dystroglycanopathy patients and animal models with abnormal glycosylation of α -DG (Yoshida-Moriguchi et al. 2010; Beedle et al. 2012; Kuga et al. 2012). In this study, using an α -DG deletion mutant (Δ mucin1-Fc) and its point mutant, we demonstrated that HNK-1ST transfers a sulfate group downstream of the phosphodiester linkage of *O*-mannose attached on the specific threonine residue where the laminin-binding glycan is modified. Although we have not yet determined the precise structure of the sulfated glycan generated on α -DG by HNK-1ST, our results revealed that HNK-1ST functions in the post-phosphoryl modification as in the case of LARGE and negatively regulates the expression of laminin-binding glycans on α -DG.

Since the originally reported enzymatic activity of HNK-1ST is to transfer a sulfate group to the C-3 position of GlcA (Bakker et al. 1997; Ong et al. 1998), the most reasonable sulfation site might be the GlcA in the repeating disaccharide unit of [-3Xyl α 1-3GlcA β 1-] generated by LARGE. 3-*O*-sulfation of the GlcA causes an inhibition of the transfer of Xyl to the C-3 position of GlcA and thereby arrests the polymerization reaction by LARGE. Our results shown here are generally consistent with this notion. For instance, HNK-1ST thoroughly arrests the laminin-binding glycan synthesis, and we detected no intermediate α -DG bearing moderately polymerized chains by the laminin overlay assay, focusing on the single action site (Figure 3). Moreover, in radiolabeled ^{35}S -sulfate group incorporation assays in this (Figure 4) and our previous study (Nakagawa et al. 2012), the HNK-1ST-dependent ^{35}S signal corresponded to the molecular weight of the protein band detected by anti-Fc antibody. In this regard, the laminin-binding form of α -DG-Fc commonly migrates more slowly on SDS-PAGE gel than the immature form detected by anti-Fc, as seen in our and other groups' studies (Figure 1-3, and Kanagawa et al. 2004; Hara et al. 2011; Nakagawa et al. 2012). These lines of evidence suggest that the laminin-binding glycan synthesis does not occur or arrests at the very initial step if the

potential sites of α -DG are sulfated by HNK-1ST. On the other hand, at the onset of the sugar polymerization by LARGE, it is not clear that LARGE transfers GlcA (or Xyl) directly to the phosphate of *O*-mannose or to unidentified linkage saccharide(s) connecting the LARGE-dependent modification to the phosphate. In the case of the biosynthesis of the glycosaminoglycan (GAG), the linear polysaccharide comprised of disaccharide repeats (GlcA and GalNAc in chondroitin sulfate or GlcA and GlcNAc in heparan sulfate) is synthesized by polymerizing enzymes (Sugahara and Kitagawa 2000), resembling the glycan structure generated by LARGE. Prior to the polymerization of the disaccharide units, the GAG-protein linkage tetrasaccharide (GlcA β 1-3Gal β 1-3Gal β 1-4Xyl) is necessary to be synthesized on carrier proteins and the GlcA in the linkage region is transferred by a different enzyme other than polymerizing enzymes (Sugahara and Kitagawa 2000). We previously reported that HNK-1ST could arrest the GAG polymerization by transferring a sulfate group to the C-3 position of the terminal GlcA of the linkage tetrasaccharide (Nakagawa et al. 2011). Considering the structural analogy between the LARGE-dependent modification and GAG, we cannot completely rule out the possibility that HNK-1ST transfers a sulfate group to the putative GlcA in the phosphodiester-linked structure on *O*-mannose prior to the action of LARGE, and arrests the subsequent polymerization by LARGE. In addition, α -DG-Fc co-expressed with HNK-1ST-EGFP in CHO-K1 cells did not react with the HNK-1 monoclonal antibody (Supplementary data, Figure S2), indicating that the reaction product synthesized by HNK-1ST on the post-phosphoryl moiety is not the typical HNK-1 carbohydrate attached on *N*-acetylglucosamine because this antibody recognizes a minimum of sulfated disaccharide (HSO₃-3GlcA β 1-3Gal) (Schmitz et al. 1994; Tokuda et al. 1998). Therefore, to address this issue, the structural determination might be the most potent method. Actually, we are trying to determine the sulfated glycan generated on α -DG by HNK-1ST using mass spectrometric analyses. However, at present, we could not detect a glycan structure containing sulfate group(s) in the glycans prepared from the recombinant α -DG-Fc co-expressed with HNK-1ST (data not shown). In addition, we were also not able to detect the phosphate group expected to present on *O*-mannose, suggesting that the post-phosphoryl moiety containing the sulfated glycan is very minor subpopulation and thus hidden by the huge amount of non-targeted glycans such as

O-mannose- or *O*-GalNAc-linked glycans. Consistent with this notion, recent glycoproteomic analyses of muscle-derived or recombinant α -DG performed by several groups showed the remarkable heterogeneity of the *O*-glycosylation of α -DG composed of various *O*-mannose- and *O*-GalNAc-linked glycans but none of them were able to detect the post-phosphoryl moiety on *O*-mannose (Nilsson et al. 2010; Stalnaker et al. 2010; Gomez Toredó et al. 2012; Harrison et al. 2012). These lines of evidence suggest the technical difficulty of the structural determination of the post-phosphoryl modification on α -DG by unknown reasons. Therefore, the structural determination of sulfated glycan(s) requires significant improvements of the preparation procedure of target glycans although it could provide valuable information about the biosynthesis of the post-phosphoryl moiety on α -DG.

In the deletion analysis of the mucin-like domain of α -DG, we showed that Δ mucin2-Fc but not Δ mucin1-Fc exhibited laminin-binding activity under conditions without LARGE overexpression and that it was derived from the post-phosphoryl modification (Figure 1B and 1C). However, we obtained an apparently contradicting result that Δ mucin1-Fc had one site to receive the LARGE-dependent modification (Figure 2B). Recently, several LARGE-dependent modification sites were identified in the N-terminal half of the mucin-like domain of α -DG including Thr317 and Thr319, present in a characteristic TPTPV sequence at the N-terminal end of the mucin-like domain (Hara et al. 2011). Considering that Δ mucin2-Fc has multiple modification sites including the TPTPV sequence whereas Δ mucin1-Fc has only one site, it might be possible that the presence of the multiple modification sites in the vicinity results in the generation of a cluster of glycans and multivalency, increasing the ligand binding affinity of α -DG. Supporting this idea, clustering of glyco-epitopes has been shown to raise the bioactivities of carrier proteins through increasing the binding affinity to their ligands, such as Tn antigen in tumor progression (Nakada et al. 1993; Matsumoto et al. 2012) and sialyl Lewis X epitope in leukocyte adhesion to endothelial cells (DeFrees et al. 1995; Malý et al. 1996).

The glycosylation pattern of α -DG varies in a tissue specific-manner (Barresi and Campbell 2006; Beedle et al. 2012; Kuga et al. 2012). Judging from previous reports, highly glycosylated forms are observed in the skeletal muscle, heart, and kidney while moderately glycosylated forms are seen in the brain, peripheral nerves, and thymus (Beedle et al. 2012;

Kuga et al. 2012). Interestingly, Kuga *et al.* demonstrated that α -DG expressed in certain wild-type mouse organs including lung and testis lacks the post-phosphoryl modification, exhibiting almost no binding affinity to the known ligands such as laminin and agrin (Kuga et al. 2012). These observations indicate that non-laminin-binding α -DG is also physiologically important and might have a different role independent of the receptor function relying on the so-called laminin-binding glycans, particularly in those organs lacking the post-phosphoryl modification. They showed that the known genes involved in the functional maturation of α -DG including *POMT1*, *POMT2*, *POMGnT1*, *Large*, *fukutin*, and *FKRP* were expressed even in organs exhibiting low glycosylation of α -DG, and thereby suggested the presence of unknown regulatory mechanisms for the post-phosphoryl modification, such as novel genes or negative regulators (Kuga et al. 2012). The tissue distribution of HNK-1 carbohydrate is restricted mainly to the brain and peripheral nerves because the expression of the glucuronyltransferases (GlcAT-P and GlcAT-S) is highly limited (Terayama et al. 1997; Seiki et al. 1999; Kizuka and Oka 2012). However, HNK-1ST is expressed in a wide range of organs (Ong et al. 1998; Tagawa et al. 2005), indicating the presence of a different function of HNK-1ST, which would be independent of HNK-1 biosynthesis. According to the previous reports, expression sites of HNK-1ST and LARGE are overlapping in several human tissues, such as brain, skeletal muscle, testis, and spleen (Ong et al. 1998; Fujimura et al. 2005). In addition, the subcellular localizations of both proteins are also overlapping in the Golgi apparatus (Supplementary data, Figure S3). Thus, our present results raise the possibility that HNK-1ST is involved in the regulation of α -DG function by suppressing the post-phosphoryl modification via antagonizing LARGE activity, especially in the organs with low glycosylation levels of α -DG. Further investigation is required to understand how HNK-1ST contributes to the functional regulation of α -DG under physiological conditions. In conclusion, our findings illustrate that a single glycosyltransferase has diverse roles that affect different glycan syntheses, implying that the glycosylation system is organized by more complicated mechanisms than previously thought.

Materials and methods

Plasmid construction

The construction of the expression plasmid for human IgG Fc fragment-fused α -DG (α -DG-Fc) was previously described (Nakagawa et al. 2012). Two deletion mutants of α -DG-Fc, Δ mucin1-Fc and Δ mucin2-Fc, were constructed as follows. For Δ mucin1-Fc, α -DG-Fc was digested with AccIII, and the longer fragment was ligated to exclude the sequence encoding Arg311 through Ile370 of α -DG. For Δ mucin2-Fc, α -DG-Fc was digested with BstEII, and the longer fragment was ligated to exclude the sequence encoding Val368 through Arg461 of α -DG. The plasmids for α -DG-T379A and Δ m1-T379A were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions with the primers CGAGGCGCCATTATTCAAGCCCCAACCCCTAGGCCCCATCCAGCC and GGCTGGATGGGGCCTAGGGTTGGGGCTTGAATAATGGCGCCTCG, employing α -DG-Fc and Δ mucin1-Fc as the templates. The plasmids encoding myc- and His-tagged mouse LARGE (LARGE-myc) and EGFP-tagged rat HNK-1ST (HNK-1ST-EGFP) were previously described (Kizuka et al. 2006; Nakagawa et al. 2012).

Cell culture and transfection

CHO-K1 and COS-1 cells were maintained in α -minimum essential medium and Dulbecco's modified Eagle medium, respectively, with 10% fetal bovine serum (FBS) in 5% CO₂ at 37°C. For cDNA transfection, cells were grown overnight and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. The culture medium was replaced with serum-free OPTI-MEM I (Invitrogen) after 5 h of incubation and collected after 48 h post transfection.

Western blotting and laminin overlay assay

Proteins were solubilized in Laemmli sample buffer, separated by SDS-PAGE using 10%

polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were incubated with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 to block nonspecific binding, followed by incubation with the primary antibodies and with HRP-conjugated secondary antibodies. Protein bands were visualized with Super Signal West Pico chemiluminescence reagent (Thermo Scientific) using a LAS-3000 Luminoimage Analyzer (FUJIFILM). The following primary antibodies were used: anti-Fc pAb (Jackson Immunoresearch); anti-EGFP mAb (Clontech); anti-myc mAb and I1H6 mAb (Millipore); anti-laminin pAb (Sigma); HNK-1 mAb (a hybridoma cell line was purchased from American Type Culture Collection). For the laminin overlay assay, nitrocellulose membranes with transferred proteins were blocked by the incubation with 5% nonfat dry milk in laminin-binding buffer (LBB; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂). After being washed in LBB, the membranes were incubated with 1 µg/ml laminin-1 (Sigma) diluted with LBB containing 3% bovine serum albumin (BSA). Bound laminin-1 was detected using anti-laminin antibody by immunoblotting as described above.

Treatment with cold aqueous hydrofluoric acid

To cleave phosphoester linkages, aqueous hydrofluoric acid (HFAq) treatment was performed as follows, according to Zhang et al. (Zhang et al. 2012) with slight modification. After SDS-PAGE, proteins were transferred onto PVDF membranes. The membranes were incubated with ice-cold 48% HFAq (Wako) at 4°C for 16 h. Control samples were prepared similarly and treated with ice-cold water instead. The membranes were then washed with ice-cold water three times to remove residual HF and subjected to laminin overlay assay or immunoblotting.

Radioactive metabolic labeling

CHO-K1 cells were grown overnight in 25 cm² cell culture flasks under normal conditions as described above. At 5 h post-transfection, the culture medium was replaced with sulfate-free M8028 MEM (Sigma), and incubation was continued for 1 h. Cells were then labeled with 30 µCi/ml of [³⁵S]-sodium sulfate (ARC Inc.). The culture medium was collected after two nights

of labeling and incubated with protein G Sepharose (GE Healthcare) for 2 h. Bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and then subjected to autoradiography or Western blotting using anti-Fc pAb.

In vitro assay for LARGE polymerization activity

LARGE-myc plasmid was transiently transfected in COS-1 cells using Lipofectamine 2000 with standard protocols. At 48 h post-transfection, the cells were lysed with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 1% Triton X-100 and protease inhibitor mixture (Nakalai Tesque), and then the cell extracts were obtained by centrifugation. The cell extracts were diluted to 5-fold volume with TBS and incubated with Ni-NTA conjugated beads (Qiagen) at 4°C for 2h. The protein-bound beads were thoroughly washed with 20 mM phosphate buffer (pH 7.4) containing 300 mM NaCl and 20 mM imidazole. LARGE-myc proteins were then eluted with 20 mM phosphate buffer (pH 7.4) containing 300 mM NaCl and 300 mM imidazole and added to the reaction mixture as the enzyme source. For the preparation of the acceptor substrates, Δ mucin1-Fc was expressed in COS-1 cells with or without HNK-1ST-EGFP and collected from the culture medium by incubation with protein G Sepharose for 2 h at 4°C. The beads were washed with PBS containing 0.1% Triton X-100 and subjected to the assays as acceptors. Polymerization reactions were performed as follows, based on Inamori et al. (Inamori et al. 2012) with several modifications. Acceptor substrate-bound beads were co-incubated with LARGE-myc proteins in reaction mixtures with a final volume of 50 μ l comprising 100 mM MOPS (pH 7.5), 10 mM MnCl₂, 10 mM MgCl₂, 1 mM UDP-glucuronic acid (GlcA), and 1 mM UDP-xylose (Xyl). Control samples were created in the same way except for omitting the donor substrates, UDP-GlcA and UDP-Xyl. After incubation at 37°C for 16 h, the acceptor substrate-bound beads were washed with PBS containing 0.1% Triton X-100, eluted by boiling in Laemmli sample buffer, and then analyzed by Western blotting.

Funding

This work was supported in part by Grant-in-Aid for Challenging Exploratory Research 23659153 (to S. O.) from JSPS and for Scientific Research on Innovative Areas 23110006 (to S.O.) from MEXT.

Abbreviations

DG, dystroglycan; ECM, extracellular matrix; ER, endoplasmic reticulum; FKR, fukutin-related protein; GlcA, glucuronic acid; GlcAT, glucuronyltransferase; HFaq, aqueous hydrofluoric acid; HNK-1ST, human natural killer-1 sulfotransferase; LARGE, like-acetylglucosaminyltransferase; MOPS, 3-(N-morpholino)propanesulfonic acid; POMT1, protein *O*-mannosyltransferase 1; POMGnT1, protein *O*-mannose β -1,2-*N*-acetylglucosaminyltransferase 1; Xyl, xylose.

References

- Bakker H, Friedmann I, Oka S, Kawasaki T, Nifant'ev N, Schachner M, Mantei N. 1997. Expression cloning of a cDNA encoding a sulfotransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope. *J Biol Chem.* 272:29942–29946.
- Barresi R, Campbell KP. 2006. Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci.* 119:199–207.
- Beedle AM, Turner AJ, Saito Y, Lueck JD, Foltz SJ, Fortunato MJ, Nienaber PM, Campbell KP. 2012. Mouse fukutin deletion impairs dystroglycan processing and recapitulates muscular dystrophy. *J Clin Invest.* 122:3330–3342.
- Beltran-Valero de Bernabe D, Currier S, Steinbrecher A, Celli J, van Beusekom E, van der Zwaag B, Kayserili H, Merlini L, Chitayat D, Dobyns WB, et al. 2002. Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am J Hum Genet.* 71:1033–1043.
- Brockington M, Blake DJ, Prandini P, Brown SC, Torelli S, Benson MA, Ponting CP, Estournet B, Romero NB, Mercuri E, et al. 2001. Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin $\alpha 2$ deficiency and abnormal glycosylation of α -dystroglycan. *Am. J. Hum. Genet.* 69:1198–1209.
- Chiba A, Matsumura K, Yamada H, Inazu T, Shimizu T, Kusunoki S, Kanazawa I, Kobata A, 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.
- DeFrees SA, Kosch W, Way W, Paulson JC, Sabesan S, Halcomb RL, Huang DH, Ichikawa Y, Wong CH. 1995. Ligand Recognition by E-Selectin: Synthesis, Inhibitory Activity and Conformational Analysis of Bivalent Sialyl Lewis x Analogs. *J Am Chem Soc.* 117:66–79.

Ervasti JM, Campbell KP. 1993. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol.* 122:809–823.

Fujimura K, Sawaki H, Sakai T, Hiruma T, Nakanishi N, Sato T, Ohkura T, Narimatsu H. 2005. LARGE2 facilitates the maturation of α -dystroglycan more effectively than LARGE. *Biochem Biophys Res Commun.* 329:1162–1171.

Gomez Toledo A, Raducu M, Cruces J, Nilsson J, Halim A, Larson G, Rüetschi U, Grahn A. 2012. O-Mannose and O-N-acetyl galactosamine glycosylation of mammalian α -dystroglycan is conserved in a region-specific manner. *Glycobiology.* 22:1413–1423.

Hara Y, Kanagawa M, Kunz S, Yoshida-Moriguchi T, Satz JS, Kobayashi YM, Zhu Z, Burden SJ, Oldstone MB, Campbell KP. 2011. Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. *Proc Natl Acad Sci USA.* 108:17426–31.

Harrison R, Hitchen PG, Panico M, Morris HR, Mekhaieel D, Pleass RJ, Dell A, Hewitt JE, Haslam SM. 2012. Glycoproteomic characterization of recombinant mouse α -dystroglycan. *Glycobiology.* 22:662–675.

Holt KH, Crosbie RH, Venzke DP, Campbell KP. 2000. Biosynthesis of dystroglycan: processing of a precursor propeptide. *FEBS Lett.* 468:79–83.

Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. 1992. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature.* 355:696–702.

Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. 2012. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. *Science.* 335:93–96.

Kanagawa M, Saito F, Kunz S, Yoshida-Moriguchi T, Barresi R, Kobayashi YM, Muschler J, Dumanski JP, Michele DE, Oldstone MB, et al. 2004. Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell*. 117:953–964.

Kizuka Y, Matsui T, Takematsu H, Kozutsumi Y, Kawasaki T, Oka S. 2006. Physical and functional association of glucuronyltransferases and sulfotransferase involved in HNK-1 biosynthesis. *J Biol Chem*. 281:13644–13651.

Kizuka Y, Oka S. 2012. Regulated expression and neural functions of human natural killer-1 (HNK-1) carbohydrate. *Cell Mol Life Sci*. in press.

Kobayashi K, Nakahori Y, Miyake M, Matsumura K, Kondo-Iida E, Nomura Y, Segawa M, Yoshioka M, Saito K, Osawa M, et al. 1998. An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature*. 394:388–392.

Kuga A, Kanagawa M, Sudo A, Chan YM, Tajiri M, Manya H, Kikkawa Y, Nomizu M, Kobayashi K, Endo T, et al. 2012. Absence of post-phosphoryl modification in dystroglycanopathy mouse models and wild-type tissues expressing non-laminin binding form of alpha-dystroglycan. *J Biol Chem*. 287:9560–9567.

Longman C, Brockington M, Torelli S, Jimenez-Mallebrera C, Kennedy C, Khalil N, Feng L, Saran RK, Voit T, Merlini L, et al. 2003. Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α -dystroglycan. *Hum Mol Genet*. 12:2853–2861.

Malý P, Thall A, Petryniak B, Rogers CE, Smith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, et al. 1996. The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell*. 86:643–53.

- Manya H, Chiba A, Yoshida A, Wang X, Chiba Y, Jigami Y, Margolis RU, Endo T. 2004. Demonstration of mammalian protein O-mannosyltransferase activity: Coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc Natl Acad Sci USA*. 101:500–505.
- Matsumoto Y, Zhang Q, Akita K, Nakada H, Hamamura K, Tokuda N, Tsuchida A, Matsubara T, Hori T, Okajima T, et al. 2012. pp-GalNAc-T13 induces high metastatic potential of murine Lewis lung cancer by generating trimeric Tn antigen. *Biochem Biophys Res Commun*. 419:7–13.
- Michele DE, Barresi R, Kanagawa M, Saito F, Cohn RD, Satz JS, Dollar J, Nishino I, Kelley RI, Somer H, et al. 2002. Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature*. 418:417–422.
- Michele DE, Campbell KP. 2003. Dystrophin-glycoprotein complex. Post-translational processing and dystroglycan function. *J Biol Chem*. 278:15457–15460.
- Muntoni F, Torelli S, Brockington M. 2008. Muscular dystrophies due to glycosylation defects. *Neurotherapeutics*. 5:627–632.
- Morita I, Kakuda S, Takeuchi Y, Kawasaki T, Oka S. 2009. HNK-1 (human natural killer-1) glyco-epitope is essential for normal spine morphogenesis in developing hippocampal neurons. *Neuroscience*. 164:1685–1694.
- Nakada H, Inoue M, Numata Y, Tanaka N, Funakoshi I, Fukui S, Mellors A, Yamashina I. 1993. Epitopic structure of Tn glycoprotein A for an anti-Tn antibody (MLS 128). *Proc Natl Acad Sci USA*. 90:2495–2499.
- Nakagawa N, Izumikawa T, Kitagawa H, Oka S. 2011. Sulfation of glucuronic acid in the linkage tetrasaccharide by HNK-1 sulfotransferase is an inhibitory signal for the expression of a chondroitin sulfate chain on thrombomodulin. *Biochem Biophys Res Commun*. 415:109–113.
- Nakagawa N, Manya H, Toda T, Endo T, Oka S. 2012. Human natural killer-1 sulfotransferase (HNK-1ST)-induced sulfate transfer regulates laminin-binding glycans on α -dystroglycan. *J Biol Chem*. 287:30823–30832.

- Nilsson J, Nilsson J, Larson G, Grahn A. 2010. Characterization of site-specific O-glycan structures within the mucin-like domain of alpha-dystroglycan from human skeletal muscle. *Glycobiology*. 20:1160–1169.
- Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell*. 126:855–867.
- Ong E, Yeh JC, Ding Y, Hindsgaul O, Fukuda M. 1998. Expression cloning of a human sulfotransferase that directs the synthesis of the HNK-1 glycan on the neural cell adhesion molecule and glycolipids. *J Biol Chem*. 273:5190–5195.
- Schmitz B, Schachner M, Ito Y, Nakano T, Ogawa T. 1994. Determination of structural elements of the L2/HNK-1 carbohydrate epitope required for its function. *Glycoconj. J*. 11:345–352.
- Seiki T, Oka S, Terayama K, Imiya K, Kawasaki T. 1999. Molecular cloning and expression of a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope. *Biochem Biophys Res Commun*. 255:182–187.
- Senn C, Kutsche M, Saghatelian A, Bösl MR, Löhler J, Bartsch U, Morellini F, Schachner M. 2002. Mice deficient for the HNK-1 sulfotransferase show alterations in synaptic efficacy and spatial learning and memory. *Mol Cell Neurosci*. 20:712–29.
- Singh J, Itahana Y, Knight-Krajewski S, Kanagawa M, Campbell KP, Bissell MJ, Muschler J. 2004. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. *Cancer Res*. 64:6152–6159.
- Stalnaker SH, Hashmi S, Lim JM, Aoki K, Porterfield M, Gutierrez-Sanchez G, Wheeler J, Ervasti JM, Bergmann C, Tiemeyer M, et al. 2010. Site mapping and characterization of O-glycan structures on alpha-dystroglycan isolated from rabbit skeletal muscle. *J Biol Chem*. 285:24882–24891.

- Sugahara K, Kitagawa H. 2000. Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. *Curr Opin Struct Biol.* 10:518–527.
- Tagawa H, Kizuka Y, Ikeda T, Itoh S, Kawasaki N, Kurihara H, Onozato ML, Tojo A, Sakai T, Kawasaki T, et al. 2005. A nonsulfated form of the HNK-1 carbohydrate is expressed in mouse kidney. *J Biol Chem.* 280:23876–23883.
- Terayama K, Oka S, Seiki T, Miki Y, Nakamura A, Kozutsumi Y, Takio K, Kawasaki T. 1997. Cloning and functional expression of a novel glucuronyltransferase involved in the biosynthesis of the carbohydrate epitope HNK-1. *Proc Natl Acad Sci USA.* 94:6093–6098.
- Tokuda A, Ariga T, Isogai Y, Komba S, Kiso M, Hasegawa A, Tai T, Yu RK. 1998. On the specificity of anti-sulfoglucuronosyl glycolipid antibodies. *J. Carbohydr. Chem.* 17:535–546.
- van Reeuwijk J, Janssen M, van den Elzen C, de Beltran-Valero BD, Sabatelli P, Merlini L, Boon M, Scheffer H, Brockington M, Muntoni F, et al. 2005. POMT2 mutations cause α -dystroglycan hypoglycosylation and Walker-Warburg syndrome. *J Med Genet.* 42:907–912.
- Yamamoto S, Oka S, Inoue M, Shimuta M, Manabe T, Takahashi H, Miyamoto M, Asano M, Sakagami J, Sudo K, et al. 2002. Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning. *J Biol Chem.* 277:27227–27231.
- Yoshida A, Kobayashi K, Many H, Taniguchi K, Kano H, Mizuno M, Inazu T, Mitsuhashi H, Takahashi S, Takeuchi M, et al. 2001. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev Cell.* 1:717–724.
- Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, Wells L, Campbell KP. 2010. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science.* 327:88–92.
- Zhang P, Hu H. 2012. Differential glycosylation of α -dystroglycan and proteins other than α -dystroglycan by like-glycosyltransferase. *Glycobiology.* 22:235–247.

Legends to figures

Figure 1. **Characteristic expression of laminin-binding glycans on the two α -DG deletion mutants.** (A) Schematic diagrams of α -DG-Fc, Δ mucin1-Fc, and Δ mucin2-Fc. α -DG is comprised of a signal peptide (SP), an N-terminal domain (N-term), a mucin-like domain (mucin-like), and a C-terminal domain (C-term). (B) α -DG-Fc, Δ mucin1-Fc, and Δ mucin2-Fc were transiently transfected with or without LARGE-myc in CHO-K1 cells. An empty vector (pEF-Fc)-transfectant was prepared as a negative control (mock). The secreted proteins were collected from the culture medium and analyzed for laminin-binding activity by laminin overlay assay and for the glycosylation state by Western blotting with IIH6 mAb (medium). Anti-Fc pAb, which recognizes the Fc fragment fused to the α -DG proteins regardless of the glycan moieties, was used to monitor the protein loading. The cell lysates were analyzed for the expression of LARGE-myc by Western blotting using anti-myc mAb (cell lysate). (C) α -DG-Fc and Δ mucin2-Fc proteins were subjected to SDS-PAGE, blotted on the membrane, and treated by cold HFAQ (HFAQ). A water-treated membrane was prepared as a control (control). The effect of the HFAQ treatment on the laminin-binding activity was evaluated by laminin overlay assay. Protein loading was monitored by Western blotting with anti-Fc pAb.

Figure 2. **Influence of the alanine-replacement of Thr379 on the functional modification of α -DG.** (A) Schematic diagrams of α -DG-T379A and Δ m1-T379A mutants. Arrowheads indicate Thr379 residue replaced with Ala. (B) CHO-K1 cells were co-transfected with LARGE-myc and α -DG-Fc, α -DG-T379A, Δ mucin1-Fc, or Δ m1-T379A. The secreted proteins were pulled down from the culture medium and analyzed by laminin overlay assay and Western blotting using IIH6 mAb and anti-Fc pAb (medium). Anti-Fc pAb was used to monitor the protein loading. CHO-K1 cell lysates were analyzed by Western blotting using anti-myc mAb to assess the expression of LARGE-myc (cell lysate).

Figure 3. **Effect of HNK-1ST on Δ mucin1-Fc with a single modification site for laminin-binding glycans.** α -DG-Fc or Δ mucin1-Fc and LARGE-myc were transiently co-expressed with or without HNK-1ST-EGFP as indicated. The secreted proteins were

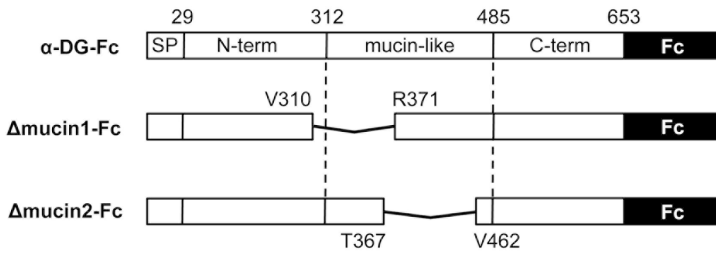
collected from the culture medium and analyzed by laminin overlay assay and Western blotting using I1H6 mAb and anti-Fc pAb (medium). Anti-Fc pAb was used to monitor the protein loading. The expression of LARGE-myc and HNK-1ST-EGFP was confirmed by Western blotting of cell lysates using anti-myc and anti-EGFP mAbs.

Figure 4. Site-specific transfer of a sulfate group by HNK-1ST. (A) Δ mucin1-Fc or Δ m1-T379A was transiently expressed with or without HNK-1ST-EGFP in CHO-K1 cells as shown. The cells were labeled with radioactive [35 S]-sodium sulfate. Δ mucin1-Fc and Δ m1-T379A were pulled down from the culture medium, separated by SDS-PAGE, and subjected to autoradiography and Western blotting using anti-Fc pAb (left). To quantify the incorporation level of the [35 S]-sulfate, the band intensity of the 35 S signal on the autoradiograph was measured. The value was normalized to the intensity of the protein band on the anti-Fc immunoblot and shown as [35 S]-sulfate incorporation (right). The graphs indicate the mean \pm s.e.m. from three independent experiments. (B) Δ mucin1-Fc co-expressed with HNK-1ST-EGFP was separated by SDS-PAGE, blotted on the PVDF membrane, and treated by cold HFAq (HFAq). A water-treated membrane was prepared as a control (control). Then, the membranes were subjected to autoradiography and Western blotting with anti-Fc pAb.

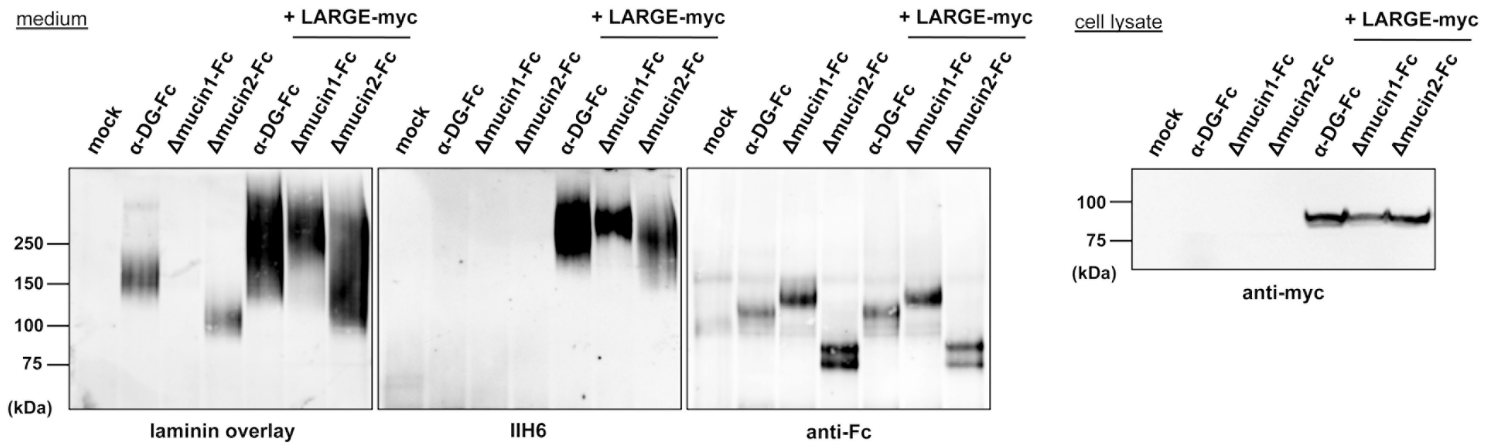
Figure 5. In vitro evaluation of LARGE polymerization activity using sulfated or non-sulfated Δ mucin1-Fc as substrates. The sugar polymerization activity of LARGE toward the sulfated α -DG was evaluated using an in vitro assay. As acceptor substrates, sulfated or non-sulfated Δ mucin1-Fc was prepared from the cells with (Δ m1+HNK-1ST) or without (Δ mucin1-Fc) co-expression of HNK-1ST-EGFP. Δ m1-T379A was prepared as a negative control. LARGE-myc proteins were co-incubated with a series of Δ mucin1-Fc protein-bound beads or beads only (control) in the absence (-) or presence (+) of UDP-GlcA and UDP-Xyl. To assess the synthesis of laminin-binding glycans, the reaction products were subjected to laminin overlay assay and immunoblotting with anti-Fc pAb.

Figure 1

A



B



C

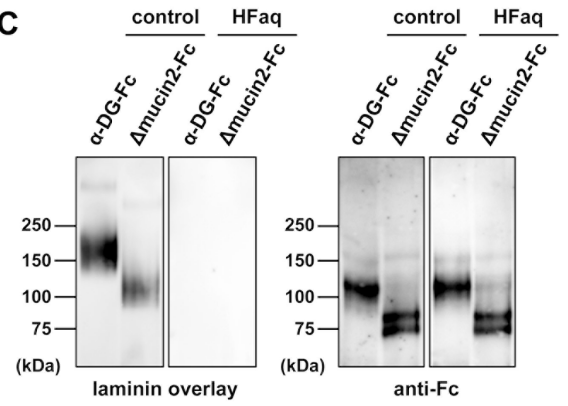
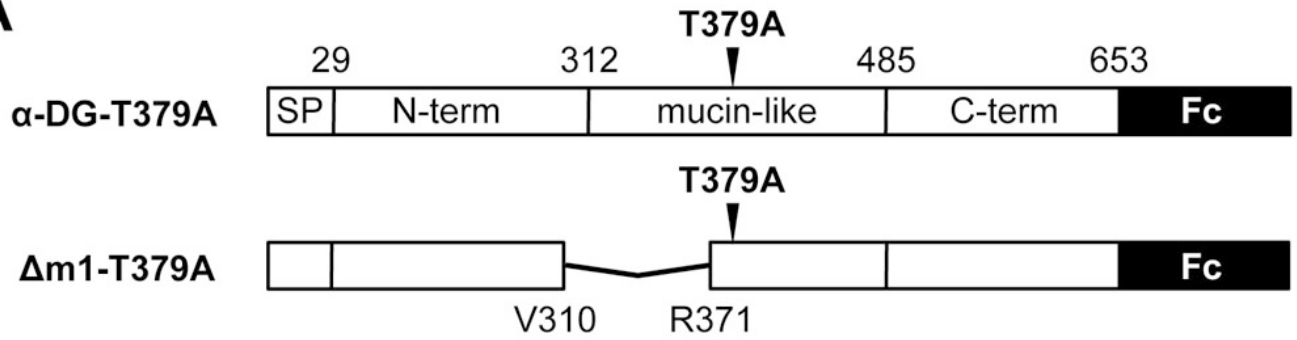


Figure 2

A



B

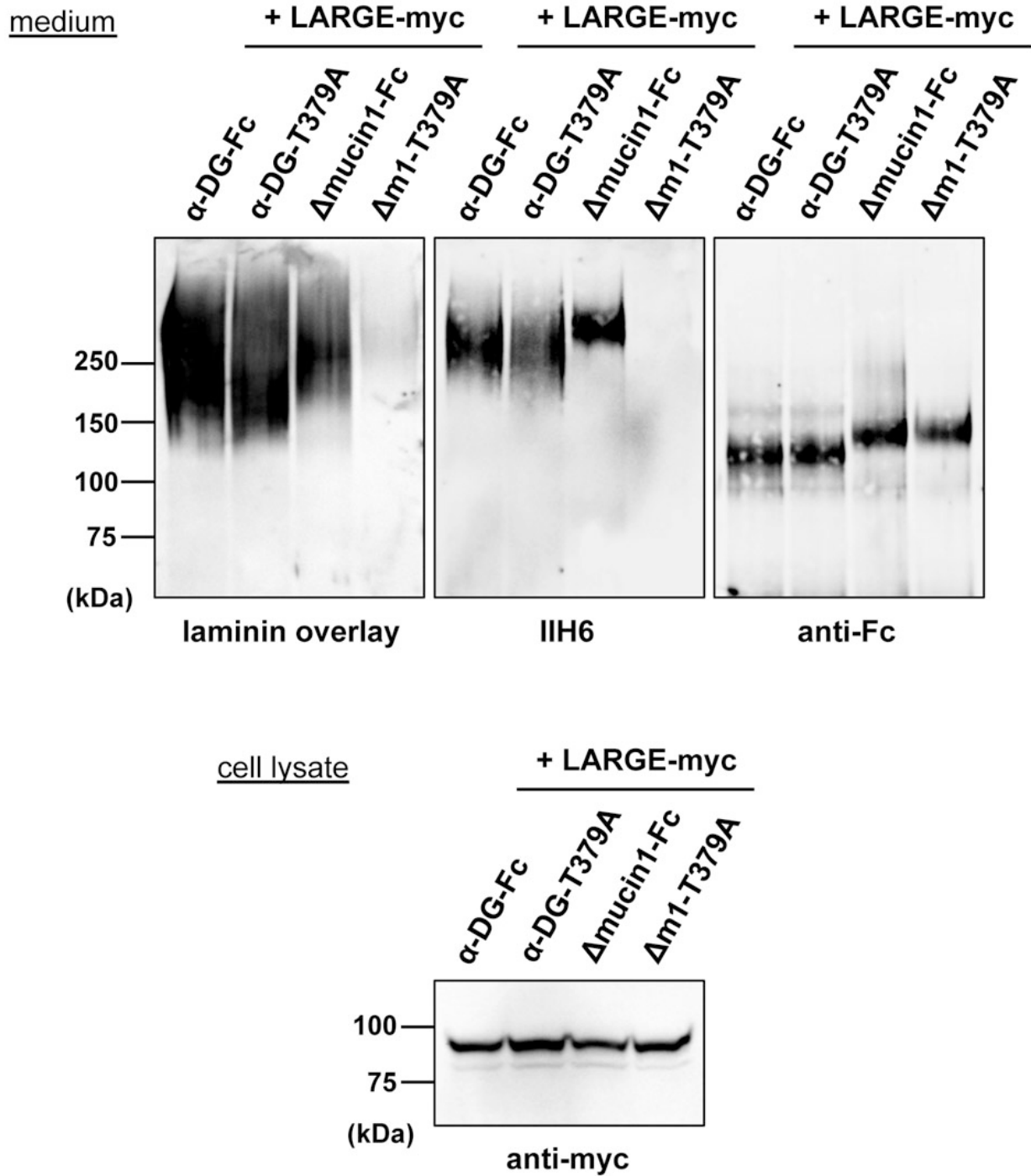
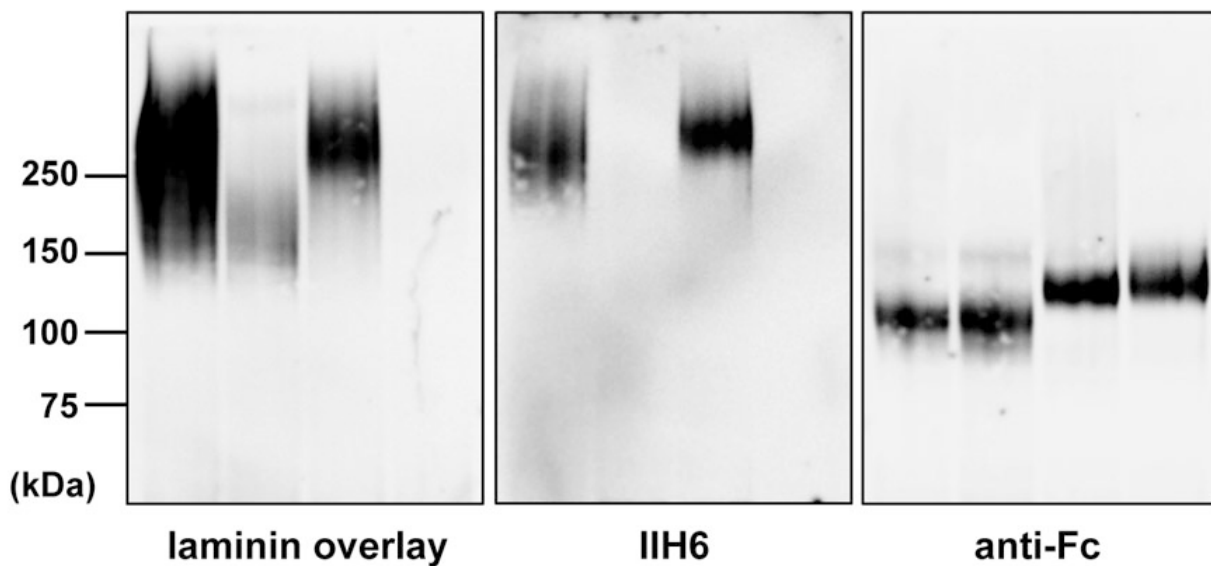


Figure 3

medium

α-DG-Fc	+	+	-	-	+	+	-	-	+	+	-	-
Δmucin1-Fc	-	-	+	+	-	-	+	+	-	-	+	+
LARGE-myc	+	+	+	+	+	+	+	+	+	+	+	+
HNK-1ST-EGFP	-	+	-	+	-	+	-	+	-	+	-	+



cell lysate

α-DG-Fc	+	+	-	-
Δmucin1-Fc	-	-	+	+
LARGE-myc	+	+	+	+
HNK-1ST-EGFP	-	+	-	+

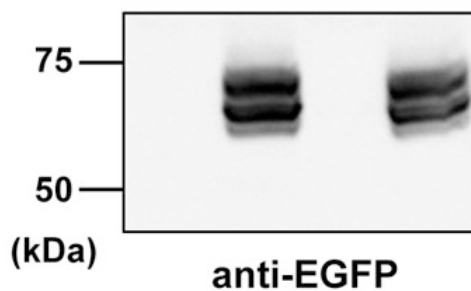
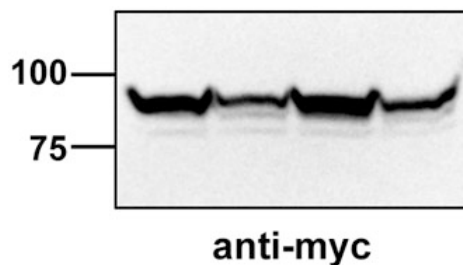
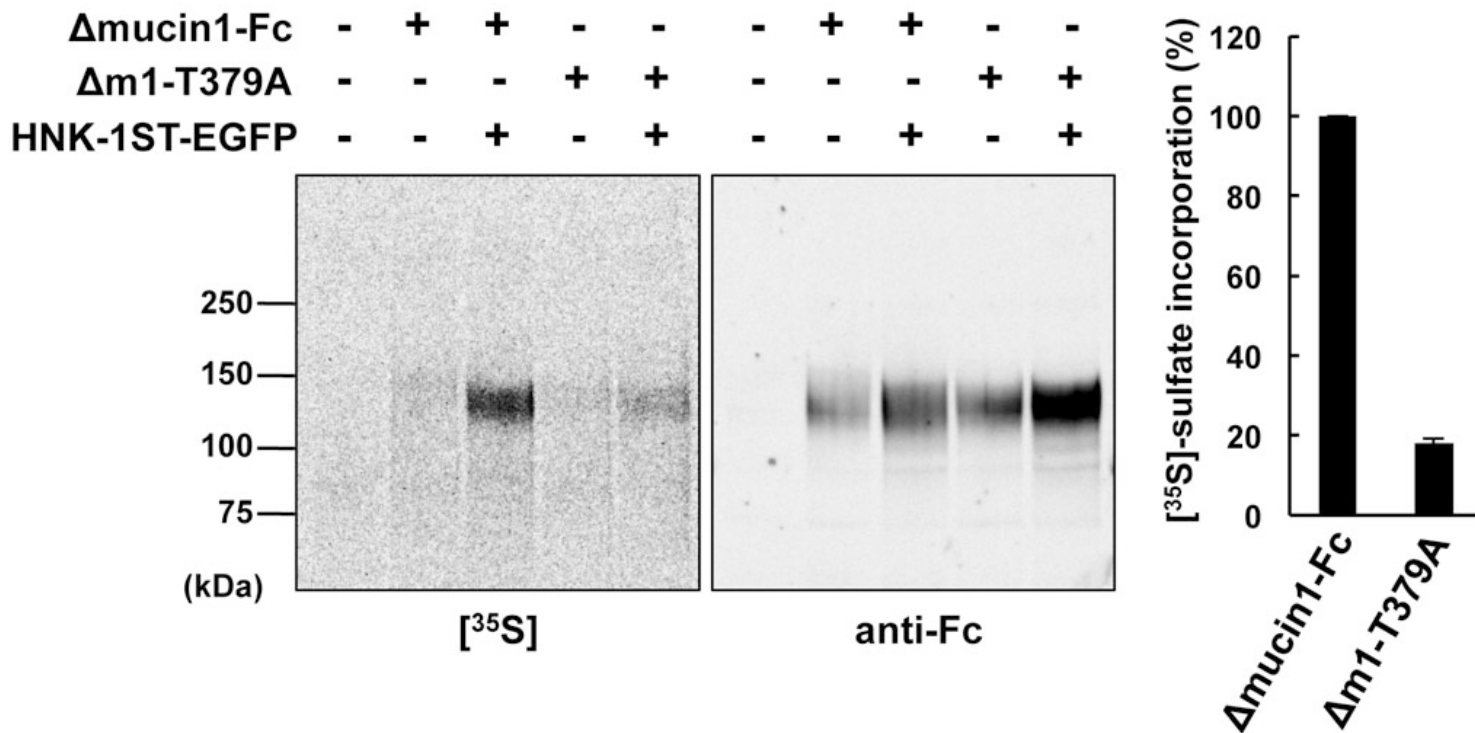


Figure 4

A



B

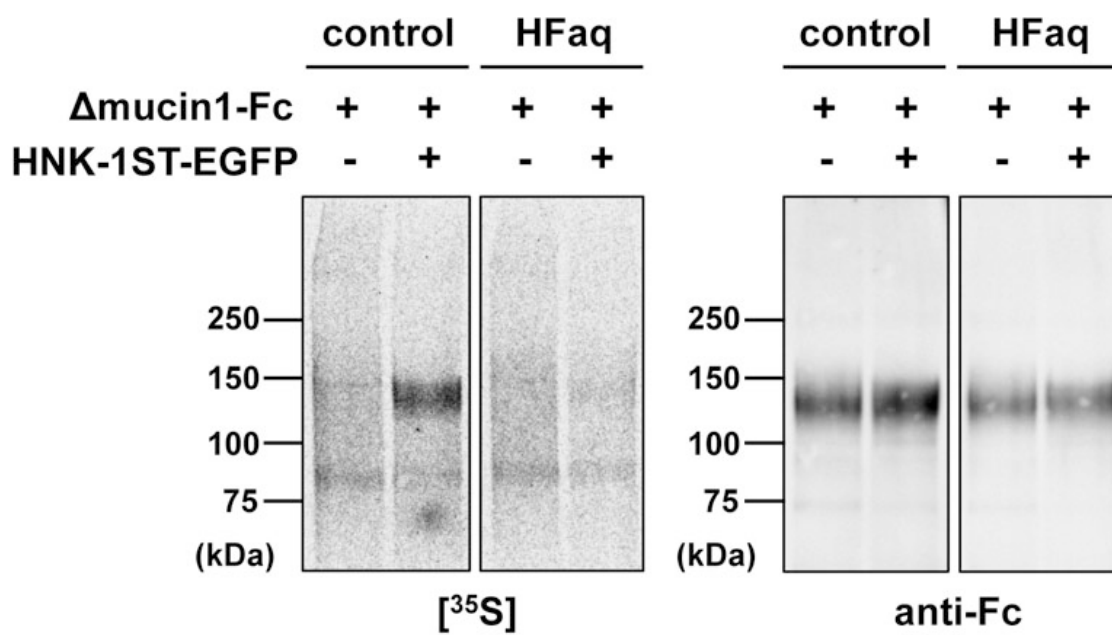
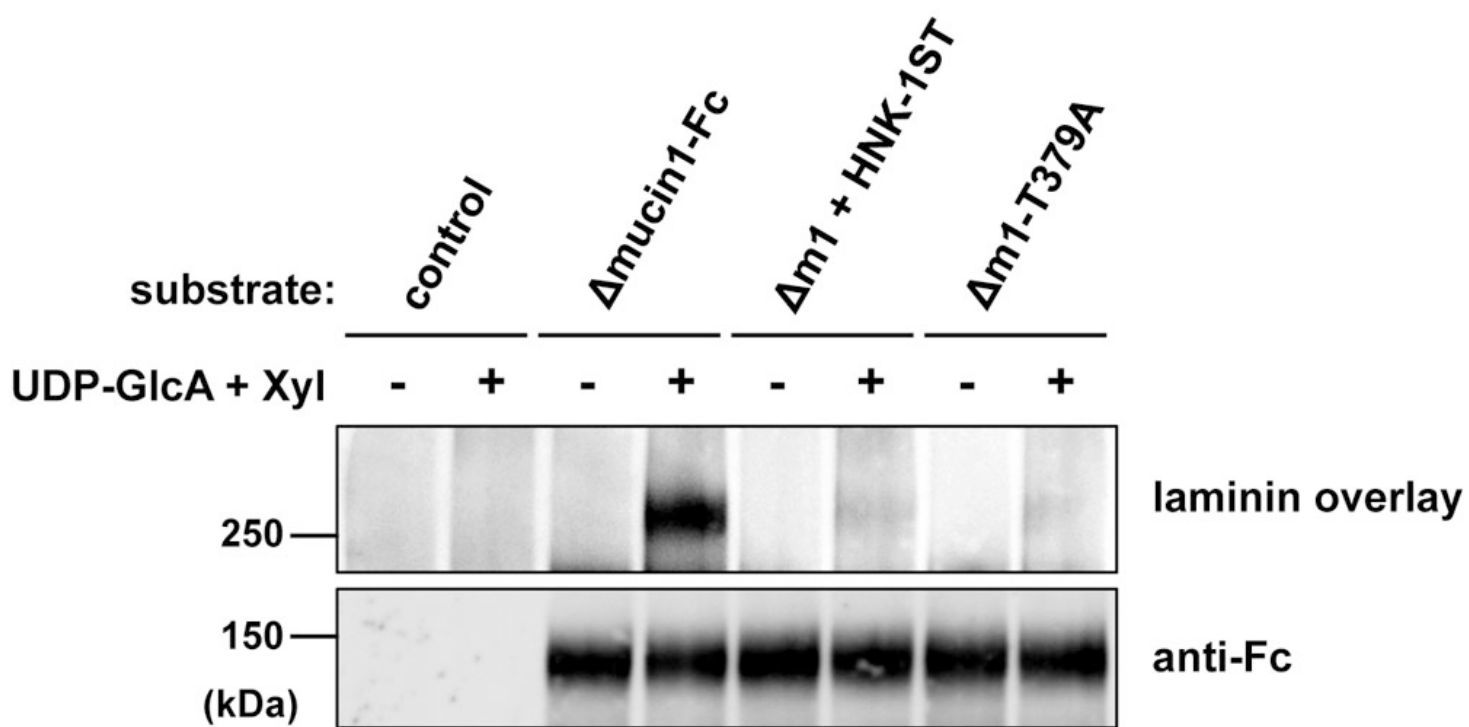
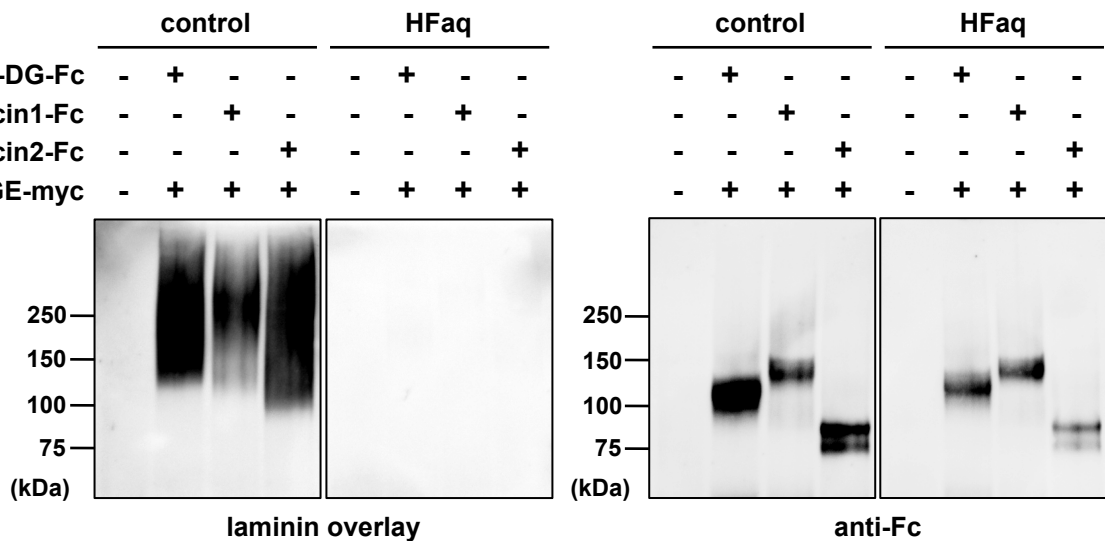


Figure 5

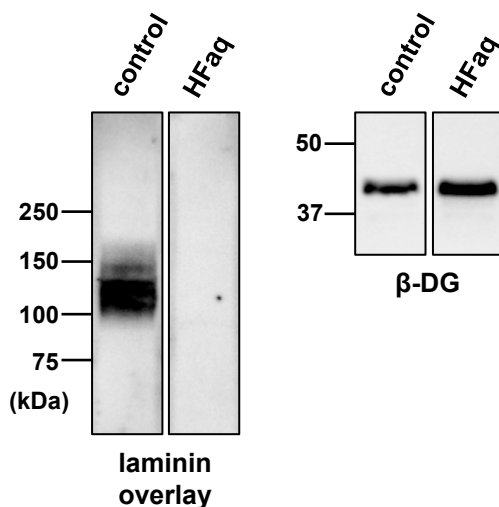


Supplemental Figure S1

A



B



C

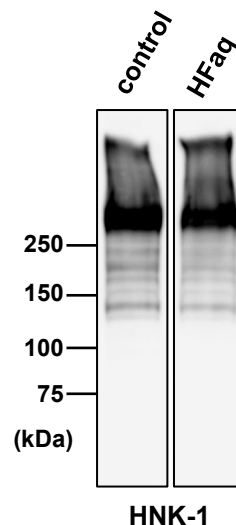


Figure S1. Effect of HF aq treatment on laminin-binding glycans and HNK-1 carbohydrate. (A) α -DG-Fc, Δ mucin1-Fc, and Δ mucin2-Fc were transiently transfected with LARGE-myc in CHO-K1 cells. α -DG-Fc and deletion mutants were collected from the culture media, subjected to SDS-PAGE, blotted on the membrane, and treated with cold HFaq (HFaq). A water-treated membrane was prepared as a control (control). The effect of the HFaq treatment on the laminin-binding activity was evaluated by laminin overlay assay. Protein loading was monitored by Western blotting with anti-Fc pAb. (B, C) Membrane fractions of two-week-old mouse brain were subjected to SDS-PAGE, transferred on the PVDF membrane, and then treated by cold HFaq (HFaq). As a control, similarly prepared membranes were treated with water (control). The effect of the HFaq treatment on the laminin-binding activity of brain α -DG was examined by laminin overlay assay (B). Equal protein loading was confirmed by immunoblotting for β -DG. The effect of the HFaq treatment on the HNK-1 carbohydrate was examined by immunoblotting with HNK-1 mAb (C). Note that the laminin-binding disappeared while the HNK-1 immunoreactivity remained in the HFaq-treated half of the blotted membrane.

Supplemental Figure S2

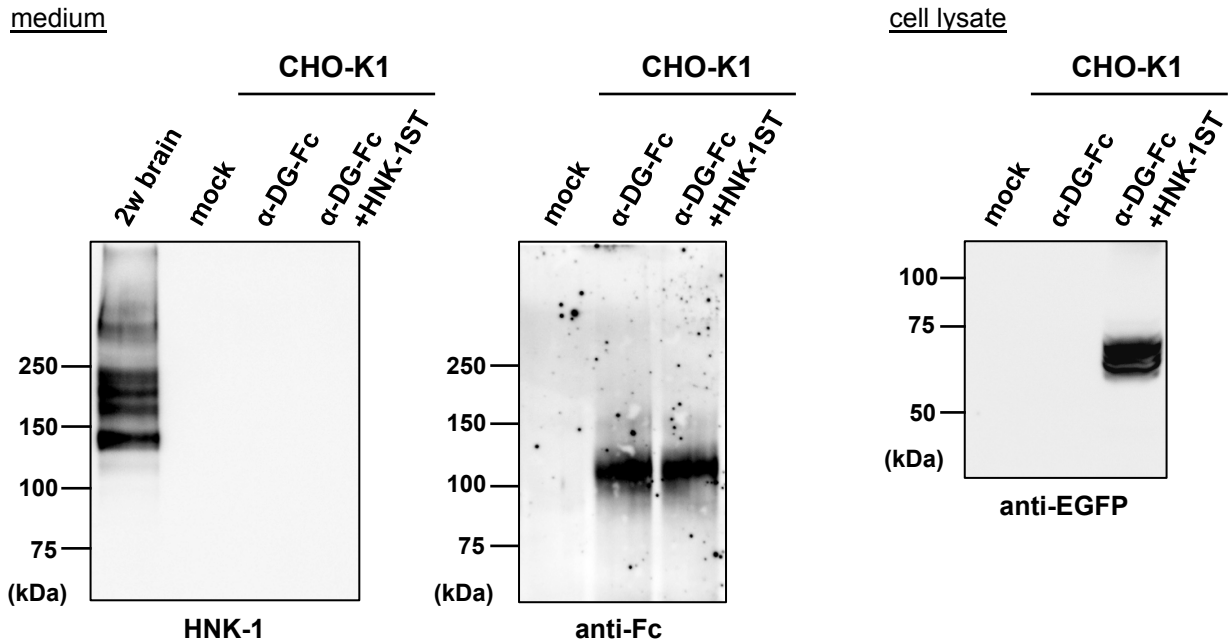


Figure S2. **Absence of the HNK-1-immunoreactivity on α -DG co-expressed with HNK-1ST.** α -DG-Fc was transiently co-transfected with HNK-1ST-EGFP in CHO-K1 cells. α -DG-Fc was collected from the culture medium, subjected to SDS-PAGE, and followed by Western blotting using the HNK-1 antibody (medium). An empty vector (pEF-Fc) was transfected as a negative control (mock). Protein loading was monitored by Western blotting with anti-Fc pAb. Membrane fractions of two-week-old mouse brain were loaded as a positive control for the HNK-1-immunoreactivity (2w brain). The expression of HNK-1ST-EGFP was confirmed by Western blotting of CHO-K1 lysates using anti-EGFP antibody (cell lysate). Note that the HNK-1-immunoreactivity was not detected on α -DG-Fc even when co-transfected with HNK-1ST-EGFP.

Supplemental Figure S3

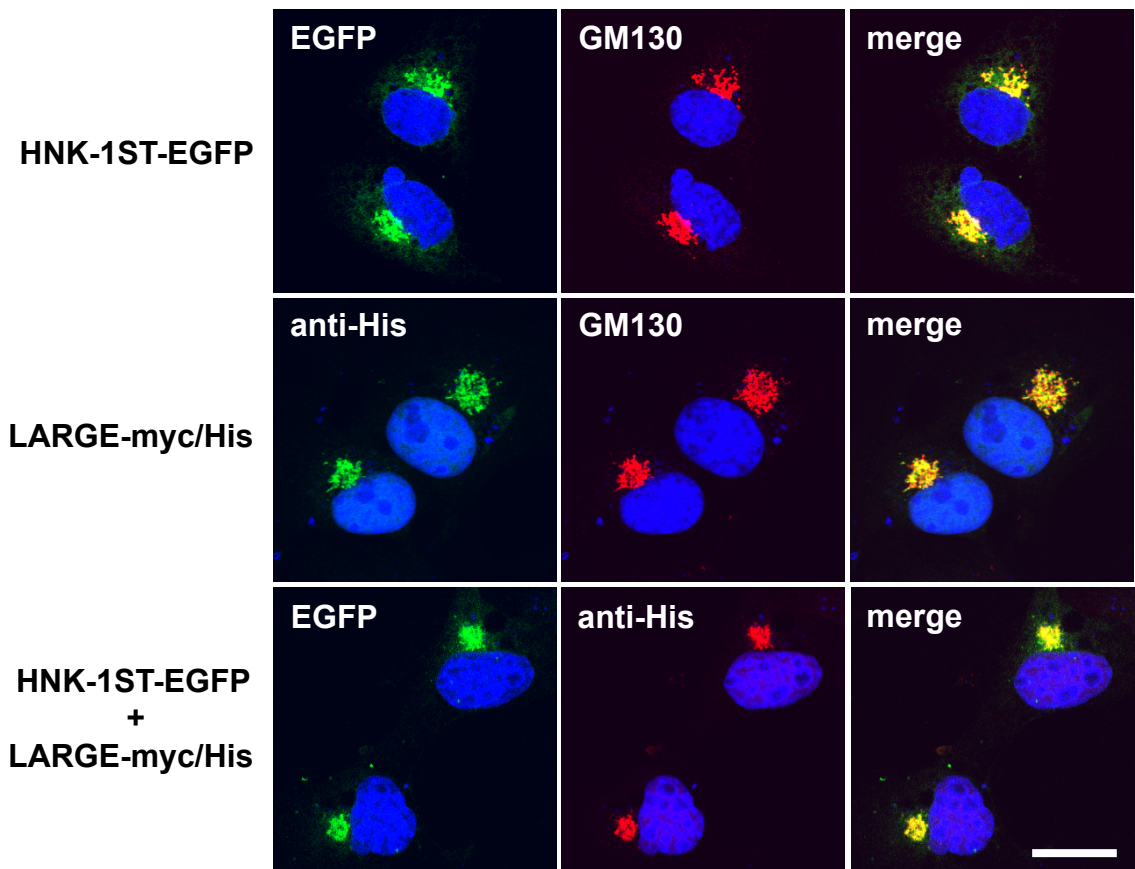


Figure S3. Subcellular localizations of HNK-1ST and LARGE.

COS-1 cells were transiently expressed with HNK-1ST-EGFP, LARGE-myc/His, or both. At 24 h post-transfection, cells were washed with PBS and fixed with 4% paraformaldehyde. To permeabilize the plasma membrane, cells were incubated with PBS containing 3% BSA and 0.1% Triton X-100. Then, cells were incubated with primary antibodies followed by Alexa Fluor 546 or 488-conjugated secondary antibodies diluted in PBS containing 3% BSA. Cells were counterstained with DAPI to label nuclei. The antibody against GM130 was used to visualize the Golgi apparatus. Scale bar, 20 μ m.