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## Structural and biochemical characterization of *O*-mannose-linked HNK-1 glycan expressed on phosphacan in developing mouse brains

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## Abstract

The human natural killer-1 (HNK-1) carbohydrate comprising a sulfated trisaccharide (HSO<sub>3</sub>-3GlcAβ1-3Galβ1-4GlcNAc-) is expressed on *N*-linked and *O*-mannose-linked glycans in the nervous system and involved in learning and memory functions. Although whole/core glycan structures and carrier glycoproteins for the *N*-linked HNK-1 epitope have been studied, carrier glycoproteins and the biosynthetic pathway of the *O*-mannose-linked HNK-1 epitope have not been fully characterized. Here, using mass spectrometric analyses, we identified the major carrier glycoprotein of the *O*-linked HNK-1 as phosphacan in developing mouse brains and determined the major *O*-glycan structures having the terminal HNK-1 epitope from partially purified phosphacan. The *O*-linked HNK-1 epitope on phosphacan almost disappeared due to the knockout of POMGnT1, an *N*-acetylglucosaminyltransferase essential for *O*-mannose-linked glycan synthesis, indicating that the reducing terminal of the *O*-linked HNK-1 is mannose. We also showed that GlcAT-P was involved in the biosynthesis of *O*-mannose-linked HNK-1 using the gene-deficient mice of GlcAT-P, one of the glucuronyltransferases for HNK-1 synthesis. Consistent with this result, we revealed that GlcAT-P specifically synthesized *O*-linked HNK-1 onto phosphacan using cultured cells. Furthermore, we characterized the as-yet-unknown epitope of the 6B4 monoclonal antibody (mAb), which was thought to recognize a unique phosphacan glycoform. The reactivity of the 6B4 mAb almost completely disappeared in GlcAT-P-deficient mice, and exogenously expressed phosphacan was selectively recognized by the 6B4 mAb when co-expressed with GlcAT-P, suggesting that the 6B4 mAb preferentially recognizes *O*-mannose-linked HNK-1 on phosphacan. This is the first study to show that 6B4 mAb-reactive *O*-mannose-linked HNK-1 in the brain is mainly carried by phosphacan.

## Introduction

Over half of the proteins in eukaryotes are covalently substituted by glycans, which play diverse roles in numerous biological phenomena (Ohtsubo and Marth 2006). The glycans on glycoproteins have been classified into two groups, an Asn-linked *N*-glycan and Ser/Thr-linked *O*-glycan. All *N*-linked glycans in mammals have the common core pentasaccharide (with or without core  $\alpha$ 1,6-fucose) structure, while the monosaccharide at the reducing end of *O*-linked glycans is diverse (including GalNAc, Xylose, Mannose, Fucose, GlcNAc, and Glucose) (Moremen et al. 2012). Among them, *O*-mannose-linked glycan, which is a rare structure in mammals, is highly enriched in the brain with 1/3 of total *O*-glycans in the brain being estimated as *O*-mannose-linked ones (Chai et al. 1999). In spite of their abundance, carrier glycoproteins and the functions of *O*-mannose-linked glycans in the brain are still poorly understood.

We previously investigated glycans in the brain with a focus on the HNK-1 carbohydrate (Kizuka and Oka 2012). HNK-1 is highly expressed in the nervous system and is functionally involved in cell adhesion, recognition, and migration (Bronner-Fraser 1987, Kunemund et al. 1988). The HNK-1 carbohydrate has a unique structure comprising a sulfated trisaccharide ( $\text{HSO}_3\text{-3GlcA}\beta\text{1-3Gal}\beta\text{1-4GlcNAc-R}$ ) (Chou et al. 1986), which is sequentially biosynthesized by the galactosyltransferase  $\beta$ 4GalT2 (Kouno et al. 2011, Yoshihara et al. 2009), one of two glucuronyltransferases (GlcAT-P and GlcAT-S), and the sulfotransferase HNK-1ST. We succeeded in cDNA cloning these HNK-1 related enzymes (Bakker et al. 1997, Seiki et al. 1999, Terayama et al. 1997) and generating GlcAT-P gene-deficient mice (Yamamoto et al. 2002). These mice were shown to almost lack expression of the HNK-1 carbohydrate in the brain, resulting in an aberration in the ability of spatial learning and memory. As cellular mechanisms, we showed that long-term potentiation in the hippocampus was significantly lower in GlcAT-P-deficient mice than in wild-type mice, which may have been due to an impairment in spine maturation and cell surface retention of glutamate receptors in developing brains (Morita et al. 2009a, Morita et al. 2009b). These functions were shown to be, at least in part, caused by a dysfunction in one of the HNK-1-carrying glycoproteins such as GluA2 or Tenascin-R (Morita et al. 2009a, Saghatelian et al. 2000), which indicated that further functional analysis of the HNK-1 glycan required the complete identification of HNK-1 carrier glycoproteins in the brain. In addition to GluA2 and Tenascin-R, several other neural cell adhesion

molecules such as NCAM, P0, and MAG have already been shown to carry HNK-1 on their *N*-glycans (Kleene and Schachner 2004), and glycan structures with the HNK-1 epitope released from these glycoproteins were determined (Kruse et al. 1984, Morita et al. 2009a, Voshol et al. 1996). However, although it has already been demonstrated that the HNK-1 carbohydrate is present in *O*-mannose-linked glycans in the brain (Yuen et al. 1997), the major carrier glycoprotein and its glycan structure with HNK-1 have not yet been clarified.

One of the soluble chondroitin sulfate proteoglycans in the brain, phosphacan, maintained HNK-1 mAb reactivity even after treatment with *N*-glycanase (Maeda et al. 1995), which indicated that this molecule was modified with HNK-1 on its *O*-glycan. Phosphacan has previously been referred to as 6B4 proteoglycan and is a secreted-type splice variant of the receptor protein tyrosine phosphatase- $\beta$ /protein tyrosine phosphatase receptor-type zeta-1 (RPTP $\beta$ /PTPRZ1). It possesses abundant chondroitin sulfate and/or keratan sulfate in the brain (Maeda et al. 1992, Maurel et al. 1994) and has diverse regulatory functions such as in neuronal migration, adhesion, and neurite promoting activity (Grumet et al. 1994, Hayashi et al. 2005, Ohyama et al. 1998, Sango et al. 2003). A recent study using knockout mice of POMGnT1, protein *O*-mannose  $\beta$ 1,2-*N*-acetylglucosaminyltransferase 1, one of the key enzymes of *O*-mannose-linked glycan biosynthesis, clearly showed that phosphacan was modified with *O*-mannose glycans as well as glycosaminoglycans (Dwyer et al. 2012). These findings suggest that phosphacan is a strong candidate for the major carrier glycoprotein of *O*-mannose-linked HNK-1 in the brain. However, the glycan structure and biosynthetic pathway of *O*-linked HNK-1 on phosphacan remain unclear.

A unique glycoform of phosphacan is also known to be recognized by the 6B4 mAb, which was originally developed against a brain-soluble proteoglycan (Maeda et al. 1995, Maeda et al. 1992). Although the 6B4 mAb has been being used to specifically detect phosphacan, the 6B4 epitope itself has still not yet been determined. Neither the polypeptide, chondroitin sulfate, nor keratan sulfate portion of phosphacan is the epitope of the 6B4 mAb (Maeda et al. 1995); however, a recent report using POMGnT1 knockout mice revealed that the epitope is included in or includes *O*-mannose-linked glycans (Dwyer et al. 2012). In addition, the epitope of another related mAb, Cat-315, which is supposed to be similar to the 6B4 mAb (Dwyer et al. 2012, Hayashi et al. 2005), was shown to partially overlap with the epitope of the HNK-1 mAb (Dino et al. 2006). Collectively, it is assumed

that the 6B4 epitope may be *O*-mannose-linked HNK-1 on phosphacan.

In the present study, we first identified the major carrier glycoprotein of the *O*-linked HNK-1 epitope as phosphacan in developing mouse brains. We also determined the *O*-linked HNK-1 structures released from partially purified phosphacan as *O*-mannose-linked glycans using mass spectrometry and POMGnT1 gene-deficient mice. By analyzing GlcAT-P gene-deficient mice and overexpressing GlcAT-P, we showed that the HNK-1 epitope was essential for recognition by the 6B4 mAb and that this mAb preferentially recognized *O*-mannose-linked HNK-1 expressed on phosphacan.

## Results

### *Expression of N-glycanase resistant HNK-1 carbohydrate in developing mouse brains*

To characterize the glycoproteins carrying the *O*-linked HNK-1 epitope, we first treated proteins from 2-week-old mouse brains with *N*-glycanase (PNGase F), and these proteins were then western blotted with the HNK-1 mAb. We simultaneously treated the samples with chondroitinase ABC (CHase ABC) to visualize the results clearly because previous reports showed that some of the HNK-1 carbohydrate was expressed on chondroitin sulfate proteoglycans (CSPGs) (Gowda et al. 1989, Krueger et al. 1992). As shown in Figure 1A, several kinds of glycoproteins in brain-soluble and membrane fractions carried the HNK-1 epitope, and a smear band was seen over 250 kDa without the CHase treatment (lanes 1 and 4). This heterogeneity in the molecular weight was due to chondroitin sulfate chains because the CHase treatment made it converge (lanes 2 and 5). Many immunoreactive bands disappeared after the PNGase F treatment, which indicated that almost all HNK-1 epitopes on glycoproteins smaller than 250 kDa were expressed on *N*-glycans (lanes 3 and 6). However, the HNK-1 epitope carried by a large CSPG molecule over 250 kDa remained even after the PNGase F treatment, indicating that this glycoprotein is the major carrier molecule of *O*-linked HNK-1. In Figure 1B, the brain-soluble fraction was treated with five kinds of glycosidases to characterize the HNK-1 carbohydrate on *O*-glycan in more detail. The results showed that the *O*-linked HNK-1 epitope on the large CSPG was not released even after sequential treatment with sialidase and *O*-glycosidase, which showed that the HNK-1 carbohydrate on this proteoglycan had a unique core glycan structure that was resistant to all glycosidases tested here. In a previous report, phosphacan purified from rat brains

remained reactive with the HNK-1 mAb after the PNGase F treatment, and the molecular weight of phosphacan after the CHase and PNGase F treatment was similar to the band shown in Figure 1B lane 4 (Maeda et al. 1995), suggesting that the major carrier for *O*-linked HNK-1 in developing brains is phosphacan. Although another CSPG, aggrecan, which has a similar molecular weight to that of phosphacan, was also reported to be reactive with the HNK-1 mAb, aggrecan was shown to be weakly expressed in developing young brains (Dino et al. 2006, Milev et al. 1998). Therefore, aggrecan is unlikely to be the major carrier molecule in the 2-week-old mouse brains shown in Figure 1. We then investigated whether phosphacan was the major carrier for *O*-linked HNK-1.

#### *Identification of phosphacan as the major carrier glycoprotein for O-linked HNK-1 and determination of the glycan structure*

To confirm that phosphacan is the major carrier for *O*-linked HNK-1, we partially purified phosphacan according to an already established method (Maeda et al. 1995). A soluble fraction (Fig. 2A, input) from 2-week-old mouse brains was applied to DEAE Sepharose (anion exchange column) to enrich CSPGs, which possess the rich negative charge of chondroitin sulfate. The eluate from DEAE Sepharose (DEAE fraction) was then treated with CHase ABC and PNGase F. The resulting proteins were stained with Coomassie Brilliant Blue (CBB) or western blotted with the HNK-1 mAb (Fig. 2A and Fig. 2B). The glycoprotein carrying the *O*-linked HNK-1 epitope over 250 kDa was highly enriched by this purification step (compare Fig. 1A, lane 1 to Fig. 2B, lane 1). After CHase and PNGase digestion, we found that only single glycoprotein was HNK-1 positive (Fig. 2B, lane 3). Together with the finding that only several kinds of proteins were visible in CBB staining after this purification (Fig. 2A, lane 3), the major carrier for *O*-linked HNK-1 was strongly suggested to be extremely concentrated in this DEAE fraction. A glycoprotein over 250 kDa was seen as an almost single band in CBB staining (Fig. 2A, arrow) whose mobility corresponded with the band shown in Figure 2B lane 3, which suggested that this protein may be the major carrier of *O*-linked HNK-1. To identify this protein, we excised the band from CBB-stained polyacrylamide gel, and the extracted protein from the band was subjected to proteomic analysis. LC/MS/MS analysis identified the protein contained in the excised gel as phosphacan. Moreover, the protein re-extracted from the excised gel piece was actually HNK-1 positive (Fig. 2C). From these results, we concluded that the major carrier

glycoprotein for *O*-linked HNK-1 is phosphacan in developing mouse brains.

We then determined the glycan structures of *O*-linked HNK-1. Since the DEAE fraction after CHase and PNGase digestion contained a single HNK-1 positive protein, namely, phosphacan (as described above), the *O*-linked glycans were released from this DEAE fraction, PA-labeled, and then analyzed by LC/MS<sup>n</sup>. Figure 3A upper panel showed the base peak chromatogram obtained by a full MS scan ( $m/z$  200–2000) using FT-ICR-MS in negative ion mode. The major molecular ions detected by a full MS scan were automatically subjected to data-dependent collision-induced dissociation-MS/MS, MS/MS/MS, and MS/MS/MS/MS. We previously determined several *N*-glycan structures having the terminal HNK-1 epitope and demonstrated that an HNK-1-containing glycan exhibited the unique HNK-1 diagnostic ion GlcA-Gal-GlcNAc<sup>+</sup> ( $m/z$  542) in the positive ion mode MS/MS spectrum (Kizuka et al. 2008, Morita et al. 2009a, Tagawa et al. 2005). According to the diagnostic ion GlcA-Gal-GlcNAc<sup>-</sup> ( $m/z$  540) in the negative ion mode, only those of oligosaccharides carrying the HNK-1 motif were sorted from all MS/MS spectra. Peaks a-e indicated the carbohydrate structure of the HNK-1 containing oligosaccharides and these were deduced from the  $m/z$  values of precursor ions detected by FT-ICR-MS and product ions obtained by MS/MS. The extracted ion chromatogram (EIC) of the ion at  $m/z$  878.2 (Peak c) is shown in Figure 3A lower panel as a representative spectrum. As typical MS/MS and MS/MS/MS spectra, the product ion spectra of peak c are shown in Figure 3B. As peaks d and e were considered to be isomers because precursor ions were observed in the same  $m/z$  value, the product ion spectra of peaks a, b, and d were shown in Supplemental Figure 1. Five kinds of HNK-1 carbohydrates were confirmed and all of these contained hexose at the reducing terminal (Fig. 3C). In the MS/MS/MS spectrum of peak a, the diagnostic ion at  $m/z$  493.4 corresponding to [NeuNAc+HexNAc]<sup>-</sup> was detected, indicating that NeuNAc is attached to HexNAc (NeuNAc-Hex-(NeuNAc-)HexNAc- or S-HexA-Hex-(NeuNAc-)HexNAc-) (Fig. 3C and Supplemental Fig. 1A). In the MS/MS/MS spectrum of peak b, the detections of the ions at  $m/z$  606.3 and 686.6 corresponding to [B<sub>3</sub>-S]<sup>-</sup> and Y<sub>2</sub><sup>-</sup> indicated that dHex is attached to HexNAc (Fig. 3C and Supplemental Fig. 1B). It should be noted here that phosphacan (RPTPβ) was known to possess the Lewis X epitope (Galβ1-4(Fuca1-3)GlcNAc-) in 2-week-old mouse brains (Nishiwaki et al. 1998, Hennen E et al. 2013), suggesting that peak b may contain both the Lewis X and HNK-1 epitopes.



### *The reducing terminal of the O-linked HNK-1 epitope on phosphacan*

The *O*-linked carbohydrate whose reducing terminal is hexose was only known as *O*-mannosylated or *O*-glucosylated glycan in mammals. However, we predicted that the reducing terminal identified here was *O*-mannose because the *O*-glucose type glycan (Xyl-Xyl-Glc) was shown to only be carried by EGF domain-rich proteins such as Notch (Rana et al. 2011) and also the HexNAc-Hex and HexNAc-(HexNAc)Hex cores in the deduced structures were completely matched with the previously identified structures (GlcNAc $\beta$ 1-2Man and GlcNAc $\beta$ 1-2(GlcNAc $\beta$ 1-6)Man) in the brain (Chai et al. 1999, Yuen et al. 1997). To investigate this possibility, we used gene-deficient mice of POMGnT1, which is one of key enzymes for *O*-mannose type glycan biosynthesis. We prepared soluble fraction from 3.5-week-old POMGnT1-deficient mouse brains with sequential digestion of CHase ABC and PNGase F, and then western-blotted with the HNK-1 mAb and anti-phosphacan antiserum, which reacted with the phosphacan polypeptide. After the digestion of CHase ABC, the HNK-1 immunoreactive band seen over 250 kDa in wild-type mice was significantly decreased in POMGnT1 knockout mice (Fig. 4A, lanes 1 and 3). The residual HNK-1 immunoreactivity in POMGnT1 knockout mice was almost completely disappeared by the PNGase F treatment (Fig. 4A, lanes 3 and 4). These results suggested that the HNK-1 epitope seen over 250 kDa was largely expressed on the *O*-mannosylated glycans and partly on the *N*-glycans. Next, we evaluated the expression levels of phosphacan in wild-type and POMGnT1 knockout mice to rule out the possibility that phosphacan was absent in POMGnT1 knockout mice. As shown in Fig. 4B, phosphacan seen over 250 kDa was expressed at comparable levels both wild-type and POMGnT1 knockout mice but the molecular weight of phosphacan was significantly decreased in POMGnT1 knockout mice. These results indicate that phosphacan in POMGnT1 knockout mice is sifted in molecular weight due to hypoglycosylation of *O*-mannose glycans and this resulted in the disappearance of the HNK-1 immunoreactivity in POMGnT1 knockout mice. Taken together, these lines of evidence indicate that major carrier of *O*-linked HNK-1 epitope is phosphacan and the epitope is expressed on *O*-mannose glycans. The anti-phosphacan-reactive band seen around 150 kDa was significantly increased in POMGnT1 knockout mice (Fig. 4B). It may be a splicing variant of phosphacan called phosphacan short isoform (PSI) (Garwood et al. 2003). Otherwise, it may be a degraded form of over 250 kDa band due to hypoglycosylation of *O*-mannose glycans because the phosphacan band seen over 250 kDa in

POMGnT1 knockout mice was slightly decreased compared to that of wild-type mice.

*GlcAT-P was essential for biosynthesis of the HNK-1 epitope on phosphacan*

Glucuronic acid transfer is the rate-limiting step during the HNK-1 epitope biosynthesis, and one of two glucuronyltransferases (GlcAT-P and GlcAT-S) is responsible for this catalysis. We previously reported that expression of the HNK-1 carbohydrate was largely absent in the brains of GlcAT-P gene-deficient mice (Yamamoto et al. 2002), which led us to speculate that the *O*-mannose-linked HNK-1 epitope on phosphacan was also biosynthesized by GlcAT-P. Soluble and membrane fractions from 2-week-old wild-type or GlcAT-P-deficient mouse brains were subjected to glycosidase digestion and western blotting (Fig. 5). The results showed that all immunoreactive bands with the HNK-1 mAb disappeared due to a deficiency in the GlcAT-P gene (Fig. 5A), which demonstrated that the *O*-mannose-linked HNK-1 epitope on phosphacan was biosynthesized by GlcAT-P as well as other *N*-linked HNK-1 epitopes. Western blotting with anti-phosphacan antiserum showed that protein expression levels of phosphacan in GlcAT-P-deficient mice were the same as those in wild-type littermates (Fig. 5B). Reactivity seen in Figure 5B in the membrane fraction was considered to be membrane-bound phosphacan and/or RPTP $\beta$ , which possessed a single transmembrane domain and an almost identical extracellular domain to phosphacan.

A previous report indicated that the Cat-315 mAb recognized the HNK-1 epitope expressed on phosphacan in developing mouse brains (Dino et al. 2006). The 6B4 mAb was also shown to recognize a unique phosphacan glycoform with *O*-mannose glycans (Dwyer et al. 2012, Maeda et al. 1995). Therefore, we speculated that the 6B4 epitope may be the *O*-mannose-linked HNK-1 epitope. To explore this possibility, brain-soluble and membrane fractions were subjected to glycosidase digestion and were detected by western blotting with the 6B4 or Cat-315 mAb (Fig. 5C and 5D). As a result, 6B4 and Cat-315 mAb reactive signals were only found over 250 kDa in both soluble and membrane fractions, and these reactivities were unchanged by PNGase F digestion. Moreover, these signals were completely lost in both fractions from GlcAT-P-deficient mouse brains. These results clearly indicated that the 6B4 mAb actually recognized the *O*-linked HNK-1 epitope as well as the Cat-315 mAb. Considering the molecular weight of phosphacan and the reactivities of the 6B4 and Cat-315 mAbs against phosphacan reported previously, Figure 5 shows that the 6B4 and Cat-315

mAbs detected *O*-linked HNK-1 on phosphacan. Interestingly, *N*-linked HNK-1 glycans on the other carrier glycoproteins were hardly detected with these antibodies. These results demonstrated that the 6B4 and Cat-315 mAbs somehow preferred the unique *O*-mannose-linked HNK-1 glycans on phosphacan.

*GlcAT-P synthesized the O-linked HNK-1 epitope on phosphacan in COS-1 cells*

To support the results presented above, phosphacan and GlcAT-P cDNAs were transfected together or alone into COS-1 cells, which did not express the HNK-1 carbohydrate endogenously. Whereas both a glucuronyltransferase (GlcAT-P or S) and sulfotransferase (HNK-1ST) are required for HNK-1 carbohydrate biosynthesis, in our previous report, only GlcAT-P overexpression in COS-1 cells allowed them to express the HNK-1 epitope, which may have been due to the endogenous expression of HNK-1ST in COS-1 cells (Terayama et al. 1997). A myc tag was fused with phosphacan at its C-terminus for detection. Secreted proteins to the culture medium from the single (GlcAT-P or phosphacan) or double (GlcAT-P and phosphacan) transfectant were precipitated and subjected to treatment with CHase ABC and PNGase F. Figure 6A lower panel showed that myc-tagged phosphacan was expressed at similar levels between single (phosphacan) (lanes 4-6) and double transfectants (lanes 7-9). By expressing GlcAT-P, cells were forced to express the HNK-1 glycan on many secreted endogenous glycoproteins (Fig. 6A upper panel, lanes 1 and 2), and all of these HNK-1 carbohydrates were removed by PNGase F digestion (Fig. 6A upper panel, lane 3). However, the double transfectant expressed PNGase F resistant HNK-1 epitopes over 250 kDa and at around 150 kDa (Fig. 6A upper panel, arrows). Since these signals were not seen in the single transfectant (compare lanes 3 and 9 in Fig. 6A upper panel), these signals were derived from overexpressed phosphacan, which demonstrated that the expressed phosphacan was modified by GlcAT-P to carry *O*-linked HNK-1 as in the brain. However, *O*-linked HNK-1 around 150 kDa was not observed in 2-week-old mouse brains (Fig. 1A). Although PSI, a splicing variant of phosphacan, was shown to be expressed in rodent brains (Garwood et al. 2003), it was unlikely that the carrier protein of *O*-linked HNK-1 at around 150 kDa (Fig. 6A, lower arrow) was PSI because our transfection experiments used full length cDNA. We speculated that it was an N-terminal fragment produced by proteolytic processing because this band was not detected by the anti-myc polyclonal antibody (pAb), but was by

anti-phosphacan antiserum (data not shown). The culture medium from the double transfectant was also sequentially treated with sialidase and *O*-glycosidase in addition to CHase and PNGase (Fig. 6B). All glycosidases tested did not reduce the immunoreactivity of phosphacan with the HNK-1 mAb, which suggested that the HNK-1 epitope on phosphacan expressed in COS-1 cells had a similar core structure to that in the brain.

Finally, immunoreactivity of the 6B4 mAb toward phosphacan expressed in COS-1 cells was investigated. Phosphacan was more strongly recognized in the brain by the 6B4 mAb than by other HNK-1 carrier glycoproteins, which suggested that the epitope of this antibody is the *O*-mannose-linked HNK-1 on phosphacan. To support this result, culture media of the transfectants were immunoblotted with the 6B4 mAb (Fig. 6C). As seen in GlcAT-P-deficient mouse brains, phosphacan without the co-expression of GlcAT-P was not recognized by the 6B4 mAb (Fig. 6C upper panel, lanes 4-6), whereas phosphacan with the HNK-1 epitopes was strongly detected with the 6B4 mAb, even after the PNGase treatment (Fig. 6C upper panel, lanes 7-9). Although other HNK-1 positive proteins were also slightly recognized by 6B4 (Fig. 6C upper panel, lanes 1-3), these signals disappeared after the removal of *N*-glycan, suggesting that the 6B4 mAb may potentially recognize *N*-linked HNK-1, at least in the overexpression experiment. Taken together, these results indicate that the 6B4 mAb is not completely specific for, but prefers the *O*-linked HNK-1 carbohydrates expressed on phosphacan to *N*-linked HNK-1 carbohydrates on other carrier proteins.

## Discussion

*N*-linked HNK-1 has been known to be expressed on glycoproteins such as P0 and NCAM and the structures of those carbohydrates have already been determined (Kruse et al. 1984, Voshol et al. 1996); however, information on the structure and biosynthesis of *O*-linked HNK-1 remains limited. Previous studies determined the core structure of *O*-linked HNK-1 as *O*-mannose glycans without identifying the carrier protein (Yuen et al. 1997). In the present study, we enriched the major carrier of *O*-linked HNK-1 from developing mouse brains and clearly identified the major carrier protein as phosphacan. Subsequent structural analysis determined the glycan structure of *O*-linked HNK-1 on phosphacan as mono- and bi-antennary *O*-mannose glycans.

The mammalian brain abundantly expresses *O*-mannose glycans, which account for ~30% of

total *O*-glycans. To date, several molecules have been identified to carry *O*-mannose glycans in the mammalian brain such as neurocan, versican,  $\alpha$ -dystroglycan, and CD24 as well as phosphacan (Bleckmann et al. 2009, Pacharra et al. 2013, Stalnakier et al. 2011b). Although some of them were shown to be HNK-1 positive, most must not be the major carrier for *O*-mannose-linked HNK-1 in the developing brain by comparing their molecular weights with our present results. Our biochemical characterization revealed that the major carrier for *O*-linked HNK-1 had a molecular weight over 250 kDa. Combining our proteomic identification with the overexpression experiments in cultured cells, we concluded that the major carrier molecule for the *O*-linked HNK-1 epitope was phosphacan in the developing mouse brain. This conclusion was confirmed by using POMGnT1 knockout mice. These results are consistent with previous studies showing that phosphacan was modified with the HNK-1 epitope and *O*-mannose glycans (Dino et al. 2006, Dwyer et al. 2012, Maeda et al. 1995). However, why phosphacan is selectively modified with HNK-1 among *O*-mannosylated proteins has yet to be determined. We previously reported that HNK-1 was biosynthesized in a well-regulated fashion by several protein complexes in living cells (Kizuka and Oka 2012). Although HNK-1 is selectively attached to limited kinds of proteins, *in vitro* enzymatic analysis of each HNK-1-related enzyme has never shown how they specifically act on the correct target proteins. Phosphacan may be endowed with a unique tertiary structure or trafficking signal within its polypeptide for preferential modification by HNK-1-related enzymes in the Golgi apparatus.

We determined the structure of the major *O*-glycans having terminal HNK-1 as mono- and bi-antennary *O*-mannose glycans using mass spectrometric analysis. A previous study demonstrated six structures of HNK-1 immunoreactive *O*-mannose glycans without sialic acid (Yuen et al. 1997). We first identified here sialylated *O*-mannose glycans with the terminal HNK-1 epitope. It is noteworthy that the HNK-1 epitope in bi-antennary structures observed in Fig. 3 peaks a, d, and e was only found in one branch, although we could not identify which branch had the HNK-1 epitope. 2,6-Branched *O*-mannose is synthesized by the distinct actions of POMGnT1 and a brain-specific *N*-acetylglucosaminyltransferase GnT-IX (Vb) (Inamori et al. 2004, Yoshida et al. 2001). Therefore, HNK-1-related enzymes may somehow co-operate with either one of the two enzymes in the Golgi apparatus. A recent study on GnT-IX-deficient mouse brains showed reduced HNK-1 levels on RPTP $\beta$ , which is a transmembrane-type splicing isoform of phosphacan (Kanekiyo et al. 2013). In

addition, we revealed that the overexpression of GnT-IX resulted in increasing the *O*-linked HNK-1 epitope on phosphacan using cultured cells (Supplemental Fig. 2). These results suggest the possibility that the HNK-1 epitope on phosphacan is *O*-mannosylated *in vitro* as well as *in vivo*, and may be preferentially expressed on the  $\beta$ 1,6-branch synthesized by GnT-IX. Since the reduced activation of astrocytes was observed in GnT-IX knockout mice after myelin injury, *O*-mannose-linked HNK-1 on phosphacan may be involved in the astrocyte activation process *in vivo*.

Phosphacan promotes neurite outgrowth depending on the cell type, such as mesencephalic neurons, cortical neurons, and hippocampal neurons from rat embryos, without being affected by CHase (Faissner et al. 1994, Garwood et al. 1999). On the other hand, phosphacan also inhibits the neurite outgrowth of retinal ganglion cells and the inhibitory effect is promoted by treatment with CHase (Inatani et al. 2001). Thus, phosphacan plays various roles in different parts of the brain, and some of these may involve *O*-mannose-linked HNK-1. Determining the carbohydrate structure of *O*-mannose-linked HNK-1 on phosphacan performed in this report would be a basis for analyzing the molecular details of phosphacan.

The most studied *O*-mannosylated glycoprotein in mammals is  $\alpha$ -dystroglycan ( $\alpha$ -DG), which is widely expressed, however, its glycosylation status differs among tissues (Barresi and Campbell 2006).  $\alpha$ -DG has been associated with its extracellular ligands, such as laminin, agrin, and pikachurin, via its *O*-mannosylated glycan (Chiba et al. 1997, Sato et al. 2008), and defects in the *O*-mannosylation of  $\alpha$ -DG was shown to cause severe muscular dystrophies, some of which exhibit brain abnormalities (Michele et al. 2002). These studies indicate that *O*-mannosylated glycans play critical roles in functioning of the muscle and nervous systems. Interestingly, a previous study reported that the HNK-1 carbohydrate was expressed on  $\alpha$ -DG (Yamada et al. 1996), even though the structure and biosynthetic pathway remain unknown. In addition, our recent report showed that one of the HNK-1-related enzymes, HNK-1ST, could regulate the ligand binding ability of  $\alpha$ -DG (Nakagawa et al. 2012). Several glycosyltransferases (including POMT1/2, and POMGnT1) involved in the *O*-mannosylation of  $\alpha$ -DG have already been identified as the genes responsible for muscular dystrophy (van Reeuwijk et al. 2005, Yoshida et al. 2001). Although  $\alpha$ -DG is not a major *O*-mannosylated protein in the brain (Stalnaker et al. 2011a), HNK-1-related enzymes may also be involved in this disease. Further analysis is required to clarify the function of the HNK-1 epitope on

$\alpha$ -DG in brain.

Knockout of POMGnT1, in which *O*-mannosylation is disturbed, also revealed the loss of the 6B4 and Cat-315 epitopes, which indicated that the epitopes of these mAbs are included in or include *O*-mannosylated glycans (Dwyer et al. 2012). Although the Cat-315 epitope was estimated to be *O*-linked HNK-1 glycan (Dino et al. 2006), there is no evidence to suggest that the 6B4 epitope overlaps with the HNK-1 epitope. In the present study using GlcAT-P-deficient mice, we clearly demonstrated that the 6B4 epitope includes the HNK-1 glycan. Moreover, previous studies and our structural analysis further indicated that the 6B4 and Cat-315 mAbs preferentially recognize the *O*-mannose-linked HNK-1 epitope on phosphacan. The major protein reactive with the Cat-315 mAb in the mouse brain was shown to switch from phosphacan (or RPTP $\beta$ ) to aggrecan along with brain development (Dino et al. 2006). Our present study demonstrated that the major carrier of *O*-mannose-linked HNK-1 is phosphacan in the developing mouse brains; however, it may be different in adult brains. It would be interesting to analyze the glycan structure of HNK-1 on aggrecan in mature brains.

This is the first report to show that the *O*-mannose-linked HNK-1 epitope is mainly carried by phosphacan in developing mouse brains and is biosynthesized by GlcAT-P. Based on these findings, future studies should clarify how this carbohydrate is specifically biosynthesized for phosphacan in the Golgi apparatus and how it functionally works to maintain the complicated network system in mammalian brains.

## Materials and methods

### *Materials*

The HNK-1 mAb (a hybridoma cell line was purchased from the American Type Culture Collection), Cat-315 mAb (Millipore), anti-myc pAb (Abcam), and anti-FLAG pAb (Sigma-Aldrich) were used as primary antibodies. The rabbit anti-GlcAT-P pAb was generated as described previously (Kizuka Y et al. 2009). Anti-phosphacan antiserum was raised in rabbits against recombinant full-length phosphacan, which was expressed in and purified from COS-1 cells. The 6B4 mAb was developed as described previously (Maeda et al. 1992). HRP-conjugated anti-mouse IgG, HRP-conjugated anti-mouse IgM, and HRP-conjugated anti-rabbit IgG (Zymed Laboratories) were used as secondary

antibodies. Chondroitinase ABC (CHase ABC) protease free and keratanase were from Seikagaku Corporation. *N*-glycosidase F (PNGase F), sialidase, and *O*-glycosidase were from Roche. The pcDNA-I/rat phosphacan plasmid was constructed as described (Nishiwaki et al. 1998). The pcDNA3.1/human GnT-IX was kindly provided from Drs. N. Taniguchi and S. Kitazume (Inamori K et al. 2003). Generation of POMGnT1-deficient mice has been described previously (Miyagoe-Suzuki Y et al. 2009).

### *Expression Plasmids*

Construction of the plasmid encoding full-length rat GlcAT-P (pIRES/P) was described previously (Kizuka et al. 2006). To express myc-tagged rat phosphacan, full-length rat phosphacan cDNA (pcDNA-I/phosphacan) was subcloned into pcDNA3.1/myc-His B (Invitrogen) as follows. First, pcDNA-I/phosphacan was digested with EcoRV and NotI, and the resulting fragment (phosphacan cDNA) was subcloned into pcDNA3.1/myc-HisB (pcDNA3.1/phosphacan), which had been digested with the same enzymes. The sequence near the 3'-end of rat phosphacan cDNA was amplified by PCR with the primers (5'-ggcactactggctctaccaac-3' and 5'-tcattcgaaacctgcctctgaactgttga-3') to create a 3'-NspV site instead of the stop codon. The amplified fragment was then digested with ApaI and NspV, and was cloned into pcDNA3.1/phosphacan, which had been digested with the same enzymes. To express FLAG-tagged human GnT-IX, full-length human GnT-IX (pcDNA3.1/human GnT-IX) was amplified by PCR with the primers (5'-aaagcgccgcatcacagtcacaccagatgg-3' and 5'-aaatctagatcacaggcagccctggcacaag-3'), digested with NotI and XbaI, and then the resulting fragment (GnT-IX cDNA) was subcloned into p3xFLAG-CMV-10 (Sigma-Aldrich), which had been digested with the same enzymes.

### *Preparation of soluble and membrane fractions from mouse brain*

A whole brain from a 2-week-old mouse was homogenized using a polytron homogenizer in 9 volumes of 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and protease inhibitors (Nakalai Tesque, Japan). The homogenate was centrifuged at 1,000 x g for 10 min to remove nuclei. The supernatant was centrifuged at 105,000 x g for 1 h. The resulting supernatant and pellet were soluble and membrane fractions, respectively.



### *Cell culture and transfection*

COS-1 cells and HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C until 50-70% confluency. Cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol after the culture medium was replaced with Opti-MEM I (GIBCO). After 6 hours of transfection, Opti-MEM I was replaced with ASF Medium 104 (Ajinomoto). Cells were incubated for another 2 or 4 days to obtain a culture medium containing secreted proteins.

### *Glycosidase digestion*

In the case of the brain-soluble fraction and culture medium, proteins were first precipitated by ethanol as follows: 2.5 volumes of ethanol were added and incubated for 20 min at -20°C. After centrifugation at 13,000 x g for 10 min, the precipitate was washed with 70% ethanol, and centrifuged again. The resulting precipitate was used as a starting material. The membrane fraction itself or ethanol-precipitated proteins were suspended with 100 mM Tris-HCl (pH 7.4) containing 30 mM sodium acetate, 5 mM EDTA, and protease inhibitors, followed by incubation with 20 mU of chondroitinase ABC (CHase) or 250 mU of keratanase for 1 h at 37°C. The reaction mixture was ethanol-precipitated again similar to above for sequential digestion with PNGase F. The resulting precipitated proteins were denatured with phosphate-buffered saline (PBS) with 0.5% SDS, 1% 2-mercaptoethanol, and 4 mM EDTA at 100°C for 5 min. The solution was then diluted with 4 volumes of PBS. After the addition of Nonidet P-40 (0.5% at the final concentration), 2 U of PNGase F was added, and the solution was incubated for 16 h at 37°C. For additional digestion with 50 mU of sialidase or 10 mU of *O*-glycosidase, these enzymes were combined with PNGase F and incubated as above.

### *SDS-PAGE and Western Blotting*

Proteins were separated by 3-10% or 5-20% gradient SDS-PAGE with the buffer system of Laemmli and were then transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.05% Tween 20, the membranes were incubated with the primary antibodies,

followed by the HRP-conjugated secondary antibodies, and protein bands were then detected with Super Signal West Pico (Pierce) using the Luminoimage Analyzer LAS-3000 (Fuji).

#### *Purification and identification of the HNK-1 carrier glycoprotein*

To identify the major carrier glycoprotein of the *O*-linked HNK-1 epitope, 10 mouse brains were excised from 2-week-old C57/BL6 mice. We purified the glycoprotein using an anion exchange column as described previously (Maeda et al. 1995) with slight modifications. Briefly, the soluble fractions were prepared, and the concentrations of NaCl and urea were then adjusted to 0.1 M and 7 M, respectively. The sample was applied to the DEAE column (DEAE Sepharose Fast Flow, GE Healthcare), which had been equilibrated with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 0.1 M NaCl, and 7 M urea. After washing with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 0.25 M NaCl, and 7 M urea, the objective glycoprotein was eluted with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 0.4 M NaCl, and 7 M urea. The eluate precipitated with ethanol was digested with CHase ABC and PNGase F as above. Purified proteins after digestion were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The objective band (~250 kDa) were excised and cut into pieces. The gel pieces were treated with 20 mM Tris/HCl (pH 8.0) containing 1% SDS to extract the glycoprotein. The extracted glycoprotein was digested with trypsin, and the peptide fragments were determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS).

#### *Protein identification by LC/MS/MS*

The tryptic digest was analyzed by LC/MS/MS using LC (Paradigm MS4 HPLC system, Michrom BioResources, Auburn, CA) and ion trap-type mass spectrometer (LTQ; Thermo Fisher Scientific, Waltham, MA). The analytical column was a reversed-phase capillary column (Magic C18, 50 × 0.2 mm, 5 μm, Michrom BioResources). The mobile phase was 0.1% formic acid containing 2% acetonitrile (A buffer) and 0.1% formic acid containing 90% acetonitrile (B buffer). The peptides were eluted at a flow rate of 2 μl/min with a gradient of 5–65% of B buffer in 50 min. The analytical conditions for MS/MS were as follows: an electrospray voltage of 2.0 kV in the positive ion mode and a collision energy of 35% for MS/MS. The spectra data obtained by MS/MS were subjected to database search analysis with the TurboSEQUENT algorithm (BioWorks; Thermo Fisher Scientific)

using the NCBI nr database. Static modification of carboxymethylation (58.0 u) at Cys was used as modified parameters for the database search analysis. The SEQUEST criterion, known as peptide probability, was set to 0.001 for identification of the proteins.

#### *Preparation of O-glycans*

The purified glycoprotein treated with CHase ABC and PNGase F to remove chondroitin sulfate and N-glycans was precipitated with ethanol, and was then lyophilized. O-glycans were released by hydrazinolysis and pyridylaminated according to the procedure described previously (Natsuka and Hase 1998) with modifications. Briefly, O-glycans were released by hydrazinolysis at 60°C for 6 h and reacylated using saturated sodium bicarbonate solution and acetic anhydride at 4°C for 30 min. After desalting with Dowex 50W-X2 (H<sup>+</sup>) (Muromachi Technos Co.) followed by lyophilization, O-glycans were pyridylaminated with 2-aminopyridine. To remove excess reagents, the reaction products were subjected to gel filtration on a column of TSK-gel G2500 PW XL (TOSOH) with 10 mM ammonium acetate (pH6.0) at a flow rate of 1 ml/min. Pyridylaminated sugar chains were collected, lyophilized, and determined by liquid chromatography/multiple stage mass spectrometry (LC/MS<sup>n</sup>).

#### *LC/MS<sup>n</sup> of O-glycans*

PA-labeled O-linked glycans were separated on a graphitized carbon column (Hypercarb, 150 × 0.075 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA) at a flow rate of 200 nL/min in a nanoFrontier nLC (Hitachi High-Tech, Tokyo, Japan). The mobile phases were 5 mM ammonium bicarbonate containing 2% acetonitrile (A buffer) and 5 mM ammonium bicarbonate containing 80% acetonitrile (B buffer). The glycans were eluted with a linear gradient of 2–80% of B buffer for 60 min. Mass spectrometric analysis was performed using a Fourier transform ion cyclotron resonance/ion trap type mass spectrometer (FT-ICR/IT-MS, LTQ-FT; Thermo Fisher Scientific). The electrospray voltage for mass spectrometry was 2.0 kV in the negative ion mode, and the collision energy was 25% for the MS/MS, MS/MS/MS, and MS/MS/MS/MS experiments. The resolution of FT-ICR-MS was 50,000, and the scan range was *m/z* 200–2,000.

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### Abbreviations

CBB, Coomassie Brilliant Blue; CHase ABC, chondroitinase ABC; CSPG, chondroitin sulfate proteoglycan; DEAE, diethylaminoethyl; GlcAT-P, glucuronyltransferase-P; GnT-IX, *N*-acetylglucosaminyltransferase-IX; HNK-1, human natural killer-1; HNK-1ST, human natural killer-1 sulfotransferase; LC/MS/MS, liquid chromatography/tandem mass spectrometry; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PNGase F, *N*-glycosidase F; POMGnT1, protein *O*-mannose  $\beta$ 1,2-*N*-acetylglucosaminyltransferase 1; RPTP $\beta$ , receptor protein tyrosine phosphatase- $\beta$ .

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## Legends to figures

**Figure 1. The *O*-linked HNK-1 epitope was mainly carried by a chondroitin sulfate proteoglycan in developing mouse brains.** (A) Soluble (lanes 1-3) and membrane fractions (lanes 4-6) of 2-week-old mouse brains were treated without (-) (lanes 1 and 4) or with (+) (lanes 2, 3, 5, and 6) chondroitinase ABC (CHase ABC) and *N*-glycosidase F (PNGase F) (lanes 3 and 6), and were then immunoblotted with HNK-1 mAb. (B) A soluble fraction of 3-week-old mouse brains with sequential digestions with CHase ABC (lanes 2-6), keratanase (lanes 3-6), PNGase F (lanes 4-6), sialidase (lanes 5 and 6), and *O*-glycosidase (lane 6) was analyzed by western blotting with the HNK-1 mAb.

**Figure 2. The glycoprotein carrying *O*-linked HNK-1 was partially purified with DEAE-Sephrose.** (A) A soluble fraction of 2-week-old mouse brains (Input) was applied to the DEAE column. The eluate (DEAE fraction, lane 1) was subjected to the CHase ABC (lanes 2 and 3) and PNGase F treatment (lane 3). Purified samples were stained with CBB. An arrow indicates the major carrier protein for *O*-linked HNK-1. (B) DEAE fractions (lane 1) with sequential digestions with CHase ABC (lanes 2 and 3) and PNGase F (lane 3) were immunoblotted with the HNK-1 mAb. (C) The protein excised from the gel ((A), arrow) was re-extracted (re-extracted fraction) and stained with CBB (left panel) or immunoblotted with the HNK-1 mAb (right panel) to confirm that the band actually contained the protein with *O*-linked HNK-1.

**Figure 3. The glycan structures of *O*-linked HNK-1 were determined.** (A) Upper panel: the basepeak chromatogram of PA-labeled *O*-linked glycans from phosphacan obtained by Fourier transform ion cyclotron resonance/ion trap mass spectrometry ( $m/z$  200–2,000) in the negative ion mode. Peaks a–e: *O*-linked HNK-1 glycans. Lower panel: the extracted ion chromatogram (EIC) of the ion at  $m/z$  878.2. (B) MS/MS and MS/MS/MS spectra of peak c ( $m/z$  878.2). (C) Deduced structures of *O*-linked HNK-1 glycans.

S, sulfate group; HexA, hexuronic acid; Hex, hexose; HexNAc, *N*-acetylhexosamine; NeuNAc, *N*-acetylneuraminic acid; Hex-PA, Hex labeled with 2-aminopyridine.

**Figure 4. The reducing terminal of the *O*-linked HNK-1 epitope on phosphacan was *O*-mannose.**

Soluble fractions prepared from 3.5-week-old wild-type (WT, lanes 1 and 2) or POMGnT1-deficient (KO, lanes 3 and 4) mouse brains were digested without (-) (lanes 1 and 3) or with (+) (lanes 2 and 4) PNGase F followed by the treatment of CHase ABC (lanes 1-4), and then immunoblotted with the HNK-1 mAb (A), anti-phosphacan antiserum (B).

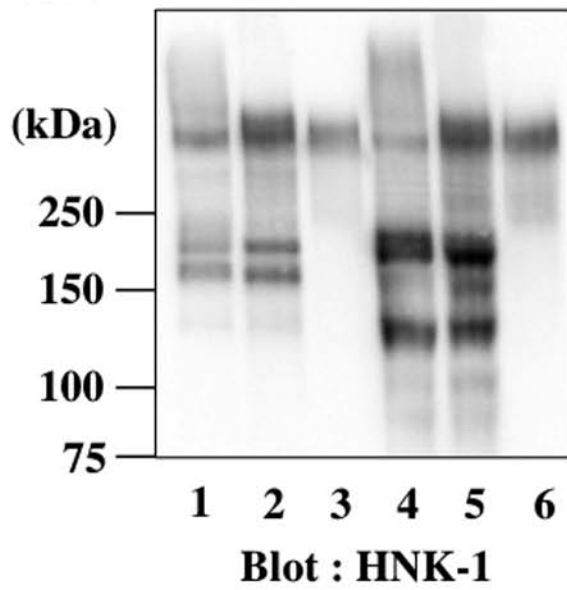
**Figure 5. The *O*-mannose-linked HNK-1 epitope was biosynthesized by GlcAT-P and preferentially recognized by the 6B4 and Cat-315 mAbs.** Soluble (lanes 1-6) and membrane (lanes 7-12) fractions of 2-week-old wild-type (WT, lanes 1-3 and 7-9) or GlcAT-P-deficient (GlcAT-P KO, lanes 4-6 and 10-12) mouse brains were treated without (-) (lanes 1, 4, 7, and 10) or with (+) CHase ABC (lanes 2, 3, 5, 6, 8, 9, 11, and 12) and PNGase F (lanes 3, 6, 9, and 12), and were then immunoblotted with the HNK-1 mAb (A), anti-phosphacan antiserum (B), 6B4 mAb (C), or Cat-315 mAb (D).

**Figure 6. Co-expression of GlcAT-P and phosphacan produced the 6B4-reactive *O*-linked HNK-1 epitope in COS-1 cells.** (A) Proteins secreted from COS-1 transfectants (single transfection of GlcAT-P (lanes 1-3), phosphacan cDNA (lanes 4-6) or double transfection of both cDNAs (lanes 7-9)) were precipitated by ethanol, and were then treated without (-) (lanes 1, 4, and 7) or with (+) CHase ABC (lanes 2, 3, 5, 6, 8, and 9) and PNGase F (lanes 3, 6, and 9). The proteins were immunoblotted with the HNK-1 mAb (upper panel) or anti-myc pAb (lower panel). Arrows indicated the full length and a cleaved N-terminal fragment of phosphacan. (B) Proteins secreted from the double transfectant were subject to sequential digestion with CHase ABC (lanes 2-5)), PNGase F (lanes 3-5), sialidase (lanes 4 and 5) and *O*-glycosidase (lane 5), and were then immunoblotted with the HNK-1 mAb. (C) Samples were prepared as in (A), and were immunoblotted with the 6B4 mAb (upper panel) or anti-myc pAb (lower panel).

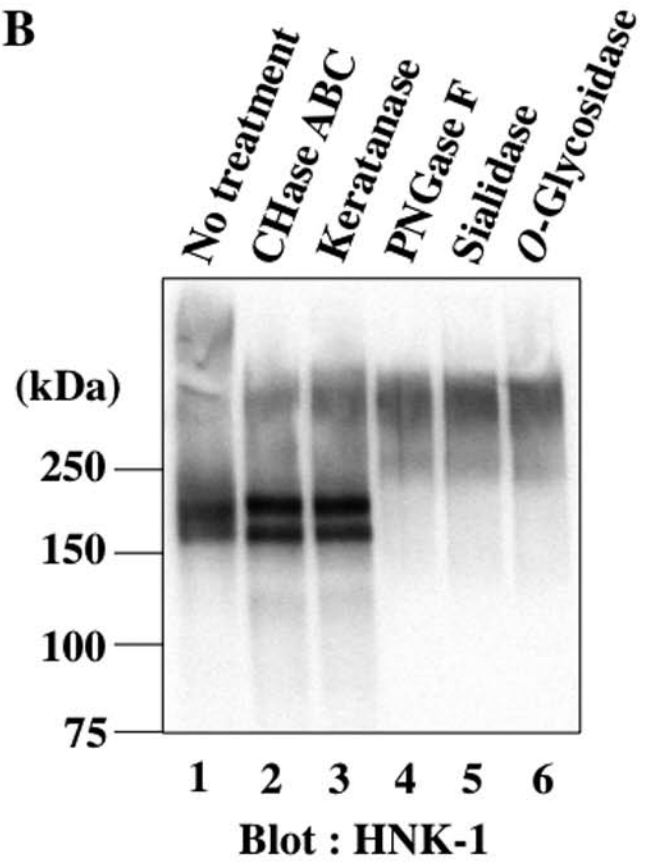
**Fig. 1**

**A**

	Soluble fraction			Membrane fraction		
CHase ABC	-	+	+	-	+	+
PNGase F	-	-	+	-	-	+



**B**



**Fig. 2**

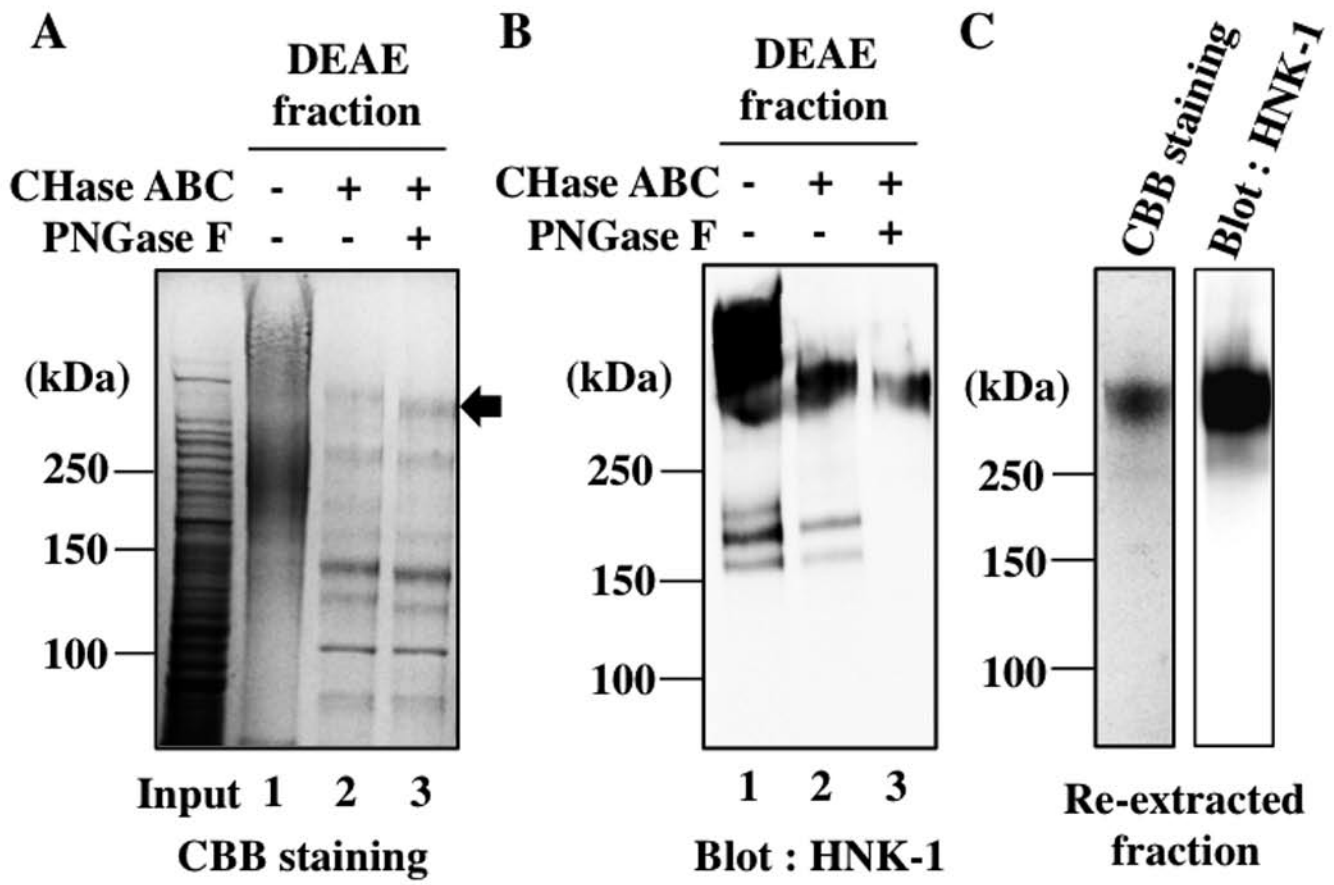
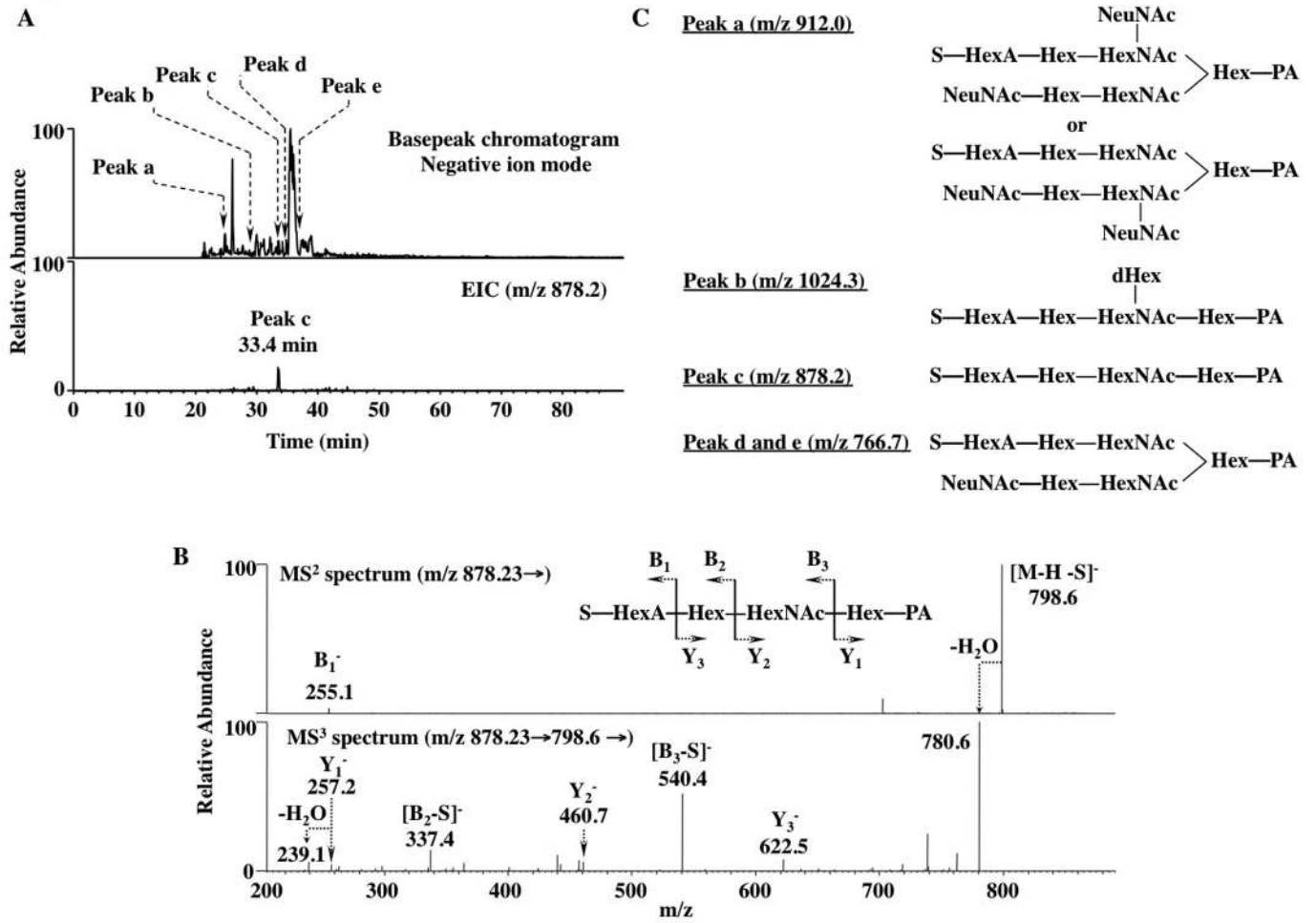


Fig. 3



**Fig. 4**

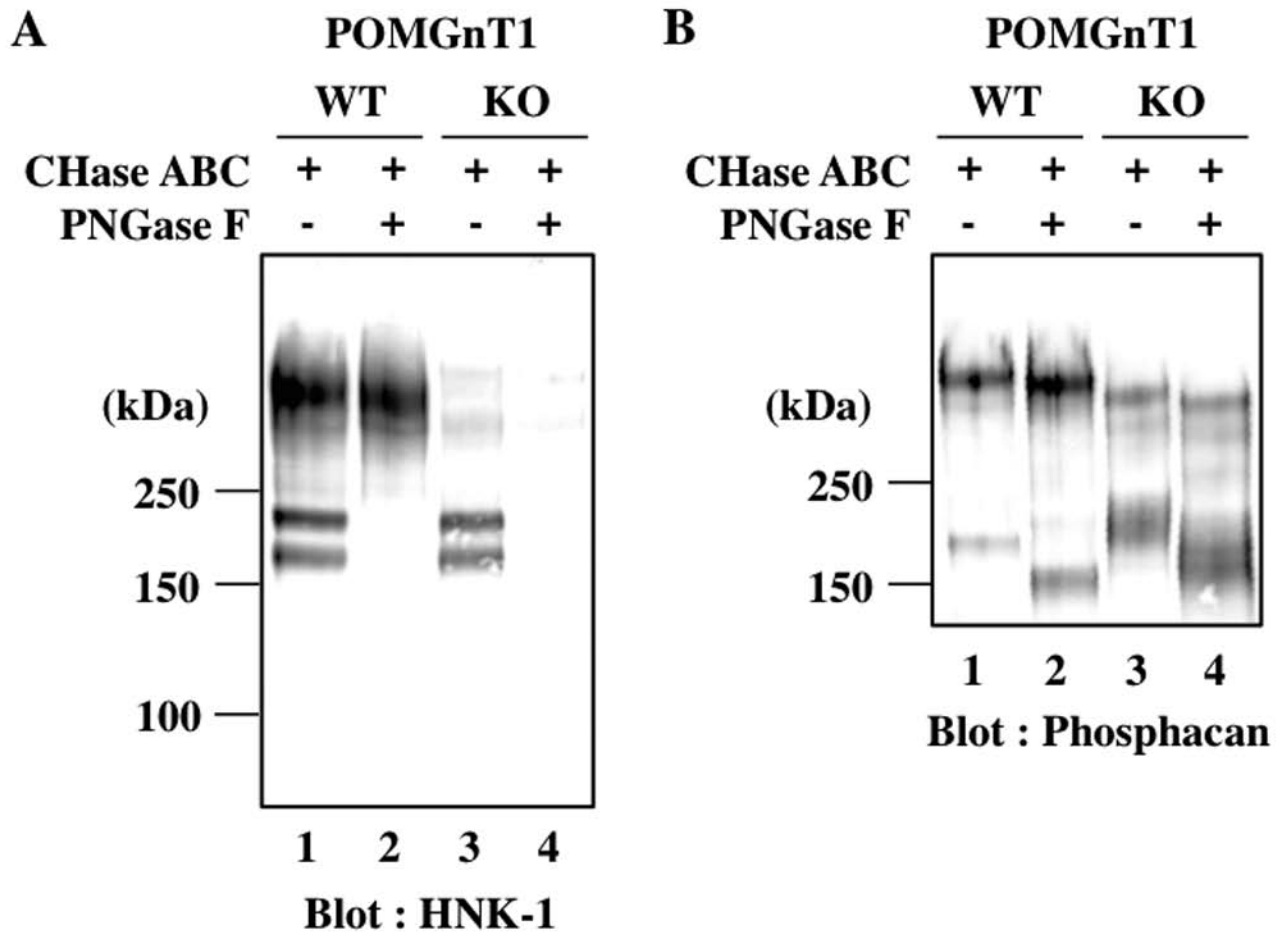
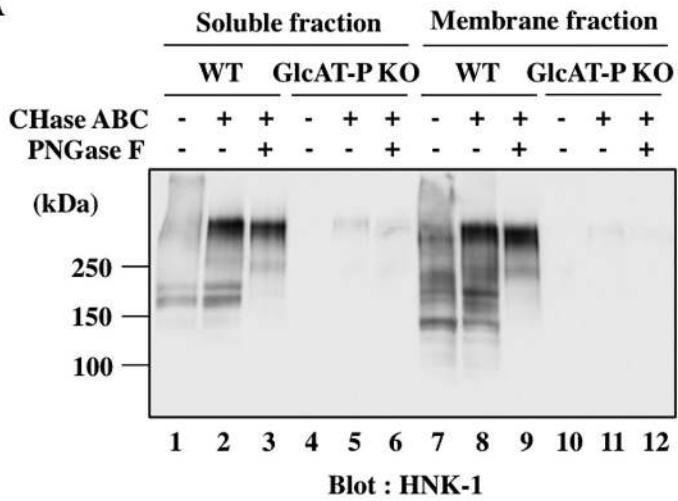


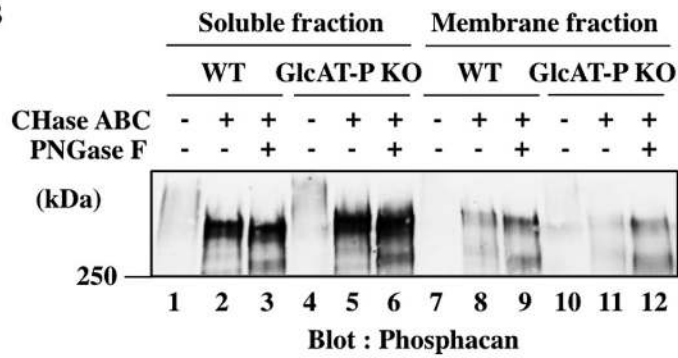


Fig. 5

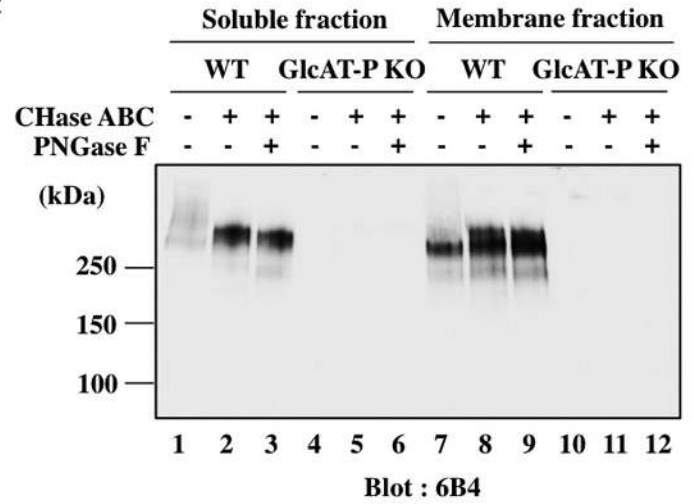
A



B



C



D

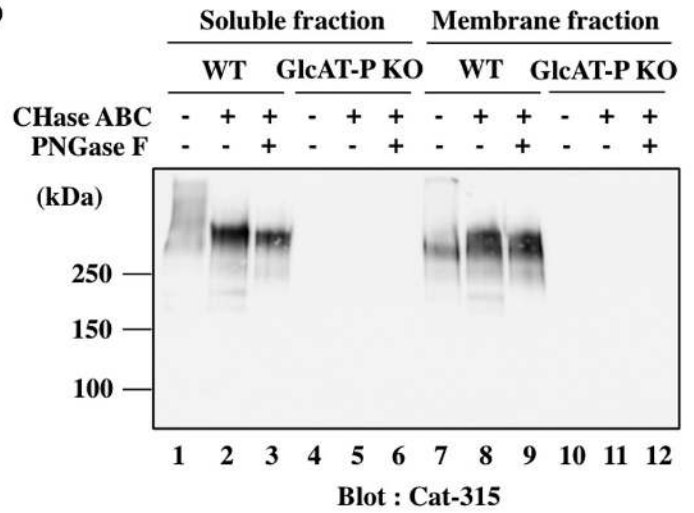
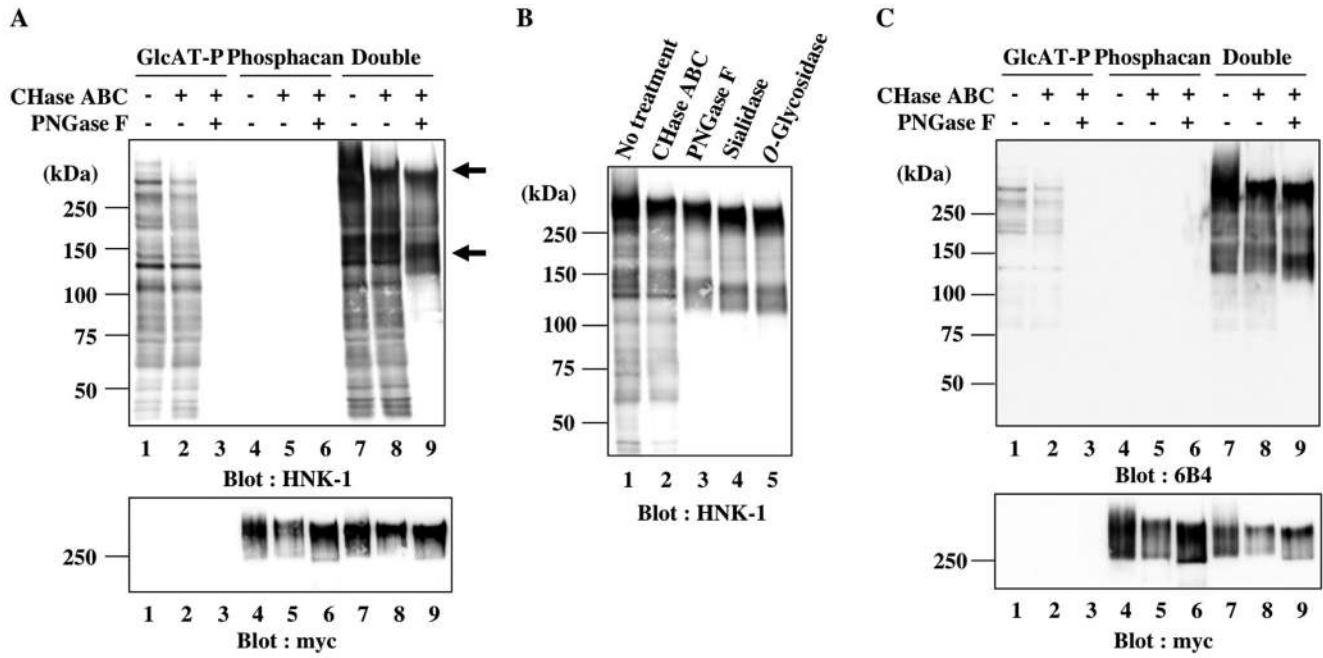
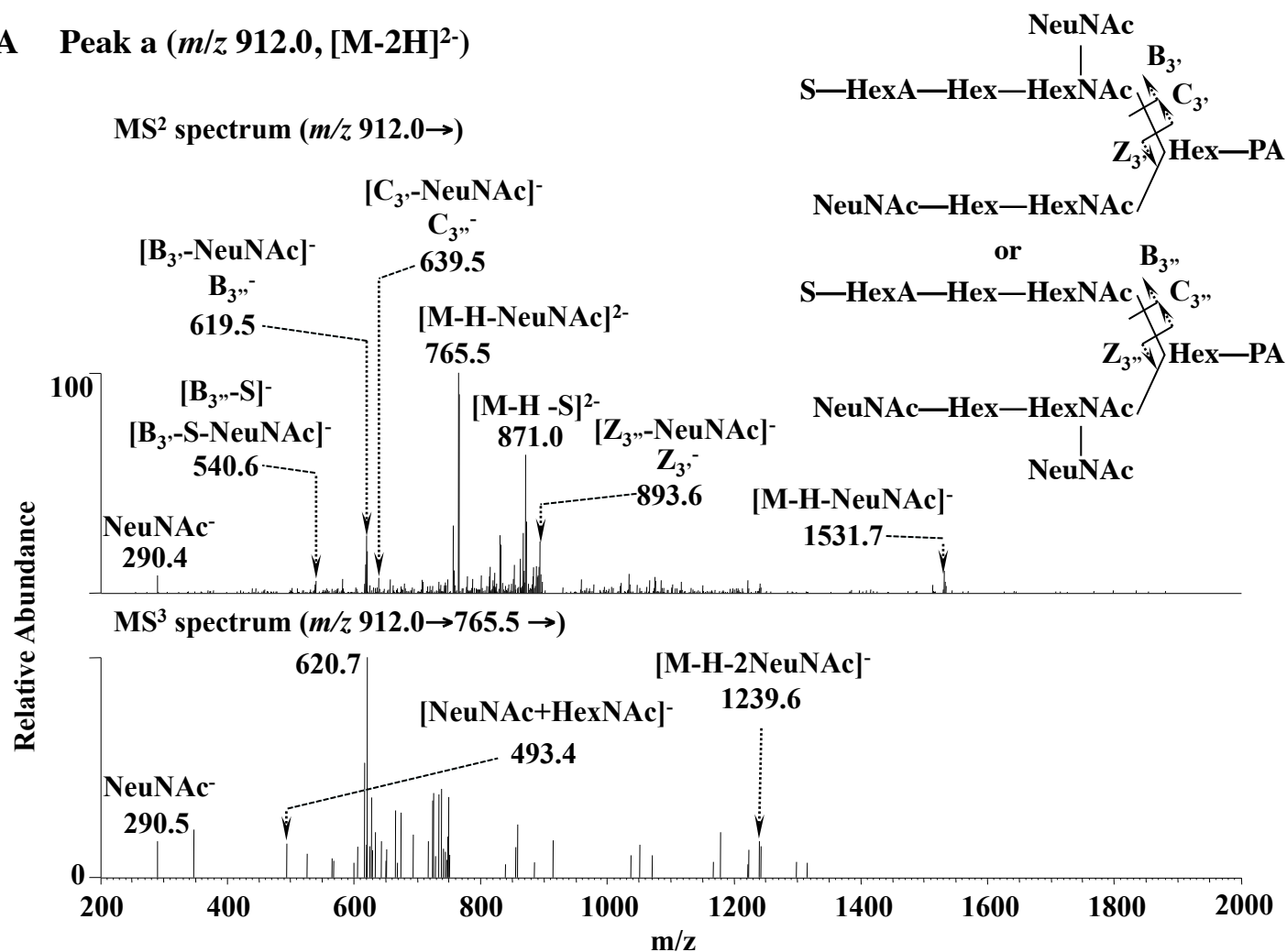


Fig. 6

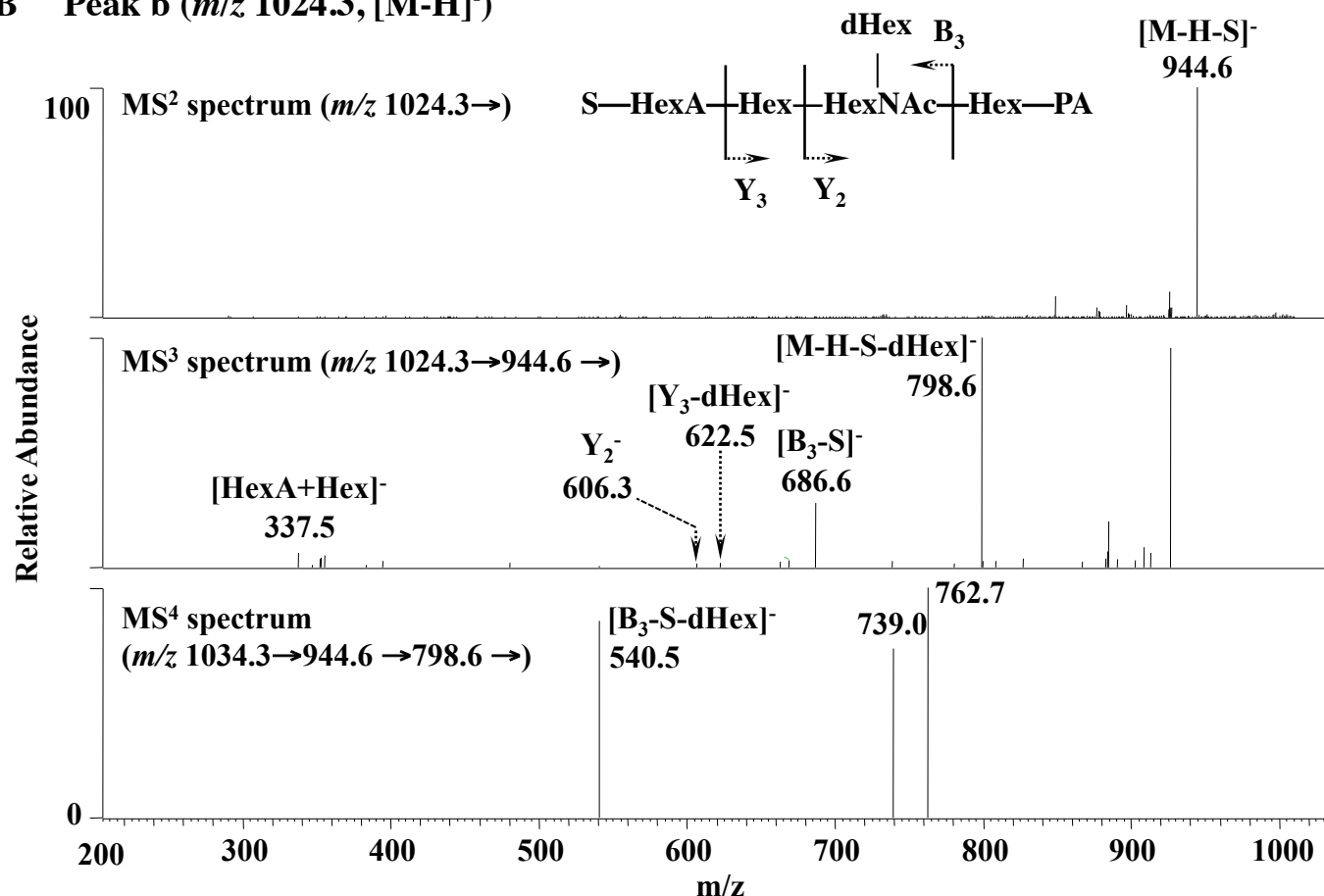


## Supplemental Fig. 1

### A Peak a ( $m/z$ 912.0, $[M-2H]^{2-}$ )

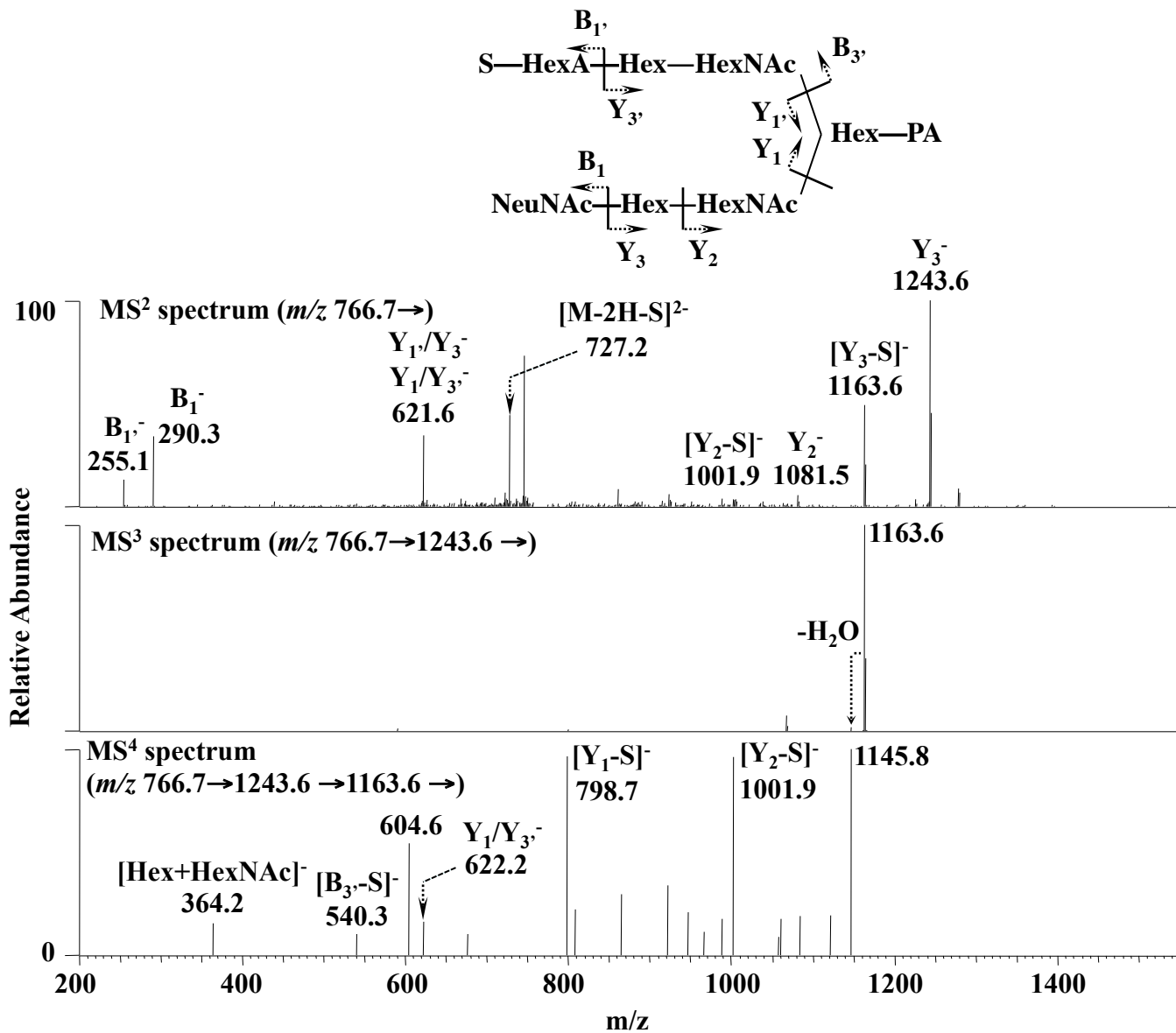


### B Peak b ( $m/z$ 1024.3, $[M-H]^{-}$ )



## Supplemental Fig. 1

### C Peak d ( $m/z$ 766.7, $[M-2H]^{2-}$ )

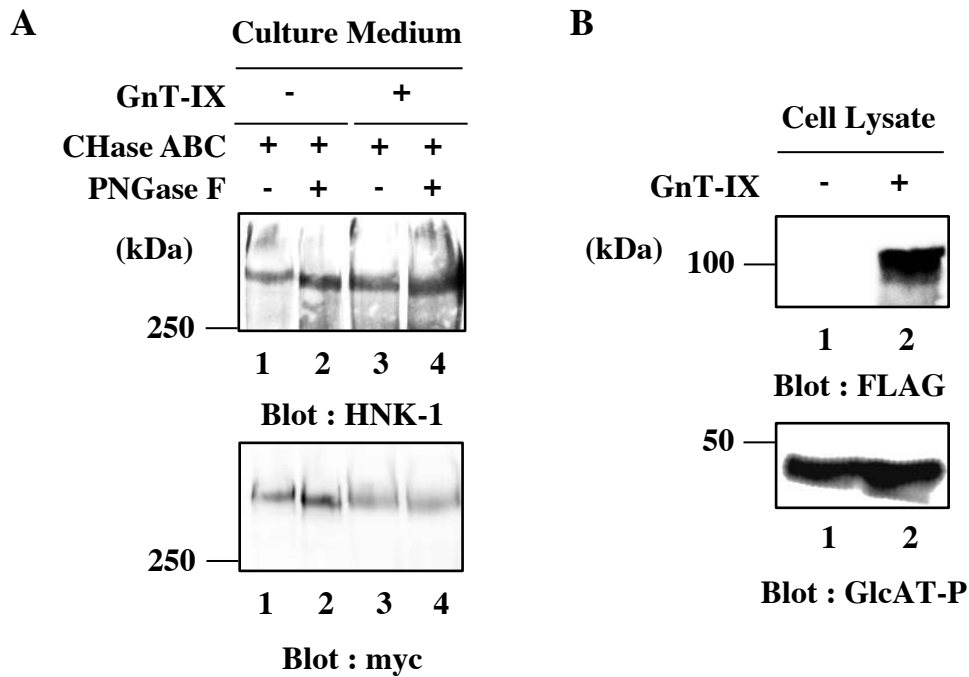


### Supplemental Figure 1. Deduced structures of *O*-linked HNK-1 glycans.

(A) MS/MS and MS/MS/MS spectra of peak a ( $m/z$  912.0). (B) MS/MS, MS/MS/MS and MS/MS/MS/MS spectra of peak b ( $m/z$  1024.3). (C) MS/MS, MS/MS/MS and MS/MS/MS/MS spectra of peak d ( $m/z$  766.7).

S, sulfate group; HexA, hexuronic acid; Hex, hexose; HexNAc, *N*-acetylhexosamine; NeuNAc, *N*-acetylneuraminic acid; Hex-PA, Hex labeled with 2-aminopyridine.

## Supplemental Fig. 2



### Supplemental Figure 2. HNK-1 epitope was synthesized on GlcNAc $\beta$ 1-6Man branch.

(A) Proteins secreted from HEK293 transfectants (double transfection of GlcAT-P and phosphacan cDNAs (lanes 1 and 2) or triple transfection of both cDNAs and GnT-IX (lanes 3 and 4)) were precipitated by ethanol. The proteins were digested without (-) (lanes 1 and 3) or with (+) (lanes 2 and 4) PNGase F followed by the treatment of CHase ABC (lanes 1-4), and then immunoblotted with the HNK-1 mAb and anti-myc pAb. Myc-tagged phosphacan was expressed at similar levels between double (lanes 1 and 2) and triple transfectant (lanes 3 and 4). The HNK-1 immunoreactivity seen over 250 kDa was increased by overexpression of GnT-IX (compared lane 1 to lane 3 or lane 2 to lane 4). (B) HEK293 transfectants (double transfection of GlcAT-P and phosphacan cDNAs (lane 1) or triple transfection of both cDNAs and GnT-IX (lane 2)) solubilized with 1% SDS were immunoblotted with anti-FLAG pAb and anti-GlcAT-P pAb.