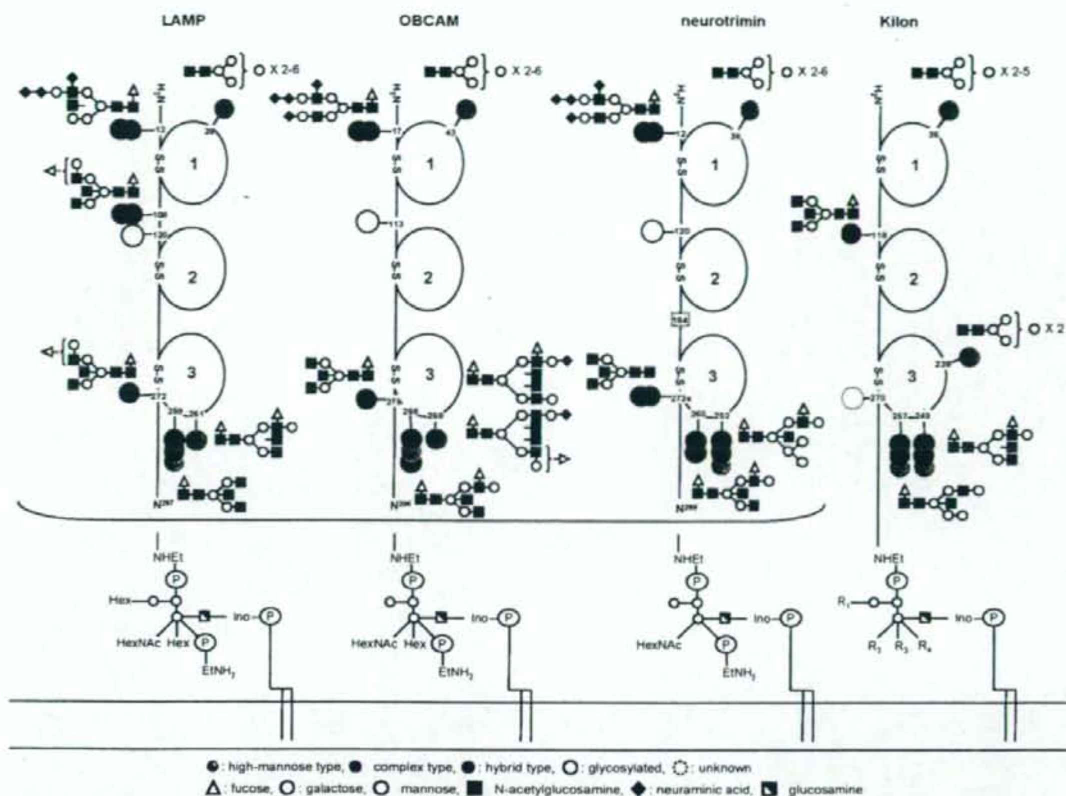

FIGURE 9: Summary of glycosylation of IgLON family proteins.

molecular ions with 81 *m/z* units intervals in the integrated mass spectrum (peaks n-1−3 in panel A2 of Figure 8) (Table 1N).

(ii) Asn118. As shown in panel B1 of Figure 8, the MS/MS/MS spectrum acquired at position 16 contained $Y_{1\alpha/1\beta}$, which suggested that the peptide portion is GTN[118]VTLTCLATGKPE. The linkage of BA-2 was deduced from the monosaccharide composition ($dHex_1Hex_3HexNAc_5$), and $Y_{1\beta/3\alpha/3\beta}{}^{2+}$ and $Y_{1\alpha}$ (inset of panel B1 of Figure 8). Additionally, the linkage of Le[a/x] or antigen H-modified and/or bisected complex type was suggested by the integrated mass spectrum (peaks o-1−5 in panel B2 of Figure 8 and Table 1O).

(iii) Asn238. The MS/MS spectra of glycopeptides that contain Asn238 were picked out from positions 22 [LFNGQQGIIIQN[238]FSTR (panel C1 of Figure 8)], 21 (RLFNGQQGIIIQN[238]FSTR), and 19 (KRLFNGQQGIII-QN[238]FSTR). These MS/MS spectra and molecular ions appearing in the integrated mass spectrum revealed that the only carbohydrate structure at Asn238 was Man-5 (peak p-1 in panel C2 of Figure 8 and Table 1P). Together with the results of the database search analysis, in which nonglycosylated peptide LFNGQQGIIIQN[238]FSTR was identified, it was suggested that Man-5 was partly attached to Asn238 (Table 1P).

(iv) Asn249. Panel D1 of Figure 8 shows the representative MS/MS spectrum of glycopeptide SILTVTN[249]VTQE at position 17. The carbohydrate structure was characterized as a Le[a/x]-modified and core-fucosylated complex type by the existence of the Le[a/x]-related ions and $Y_{1\alpha}$. The integrated mass spectrum and alternative LC−MS^n with the C30 column (scan ranges of *m/z* 700−2000 and 1000−2000) suggested that Asn249 is glycosylated with Le[a/x] or antigen H-modified core-fucosylated hybrid- and complex-type oligosaccharides, BA-2, and Man-5 (peaks q-1−11 in panel D2 of Figure 8 and Table 1Q).

(v) Asn257. As shown in panel E1 of Figure 8, one of the glycopeptides eluted at position 10 was identified as HFGN[257]YTCVAANK linked by $dHex_1Hex_5HexNAc_4$ based on $Y_{1\alpha/1\beta}$ ion in the MS/MS/MS spectra and monoisotopic mass. The carbohydrate structure was characterized as a bisected- and core-fucosylated hybrid-type oligosaccharide based on the presence of $Y_{1\beta/3\alpha/3\beta}{}^{2+}$ and $Y_{1\alpha}$ (inset of panel E2 of Figure 8). Other major glycans were estimated as Man-5, Le[a/x]-modified complex- and hybrid-type oligosaccharides, and BA-2 (peaks r-1−7 in panel E2 of Figure 8 and Table 1R).

DISCUSSION

The cell adhesion molecules in the central nervous system play an essential role in the differentiation of neuronal cells and formation of neural circuits. Although glycosylation on the cell adhesion molecules is known to regulate cell−cell interactions (*2−4*), their carbohydrate structures remain unknown due to the difficulty with respect to their isolation and the limited sample amounts. The glycans in the IgLON family proteins are considered to be implicated in the

formation of neural circuits, including migration of neuronal cells, axonal guidance, and fasciculation. However, the high degree of homology of their amino acid sequences makes it difficult to isolate them from each other and to analyze their carbohydrate structures in detail.

In this study, we performed a site-specific glycosylation analysis of LAMP, OBCAM, neurotrimin, and Kilon simultaneously using SDS−PAGE and LC−MS^n. Enriched GPI-linked proteins were separated by SDS−PAGE, and four target proteins were extracted from a gel piece together with other contaminating proteins. The protein mixture was digested and analyzed by the C30 and C18-LC−MS^n runs via MS, data-dependent MS in SIM by the FT ICR-MS, and data-dependent MS/MS and MS/MS/MS. A set of MS data consisting of the mass spectrum, the mass spectrum acquired by the FT ICR-MS in SIM mode, the data-dependently acquired MS/MS, and the MS/MS/MS spectra of a glycopeptide was selected from all MS data on the basis of the existence of the oligosaccharide characteristic oxonium ions in the MS/MS spectrum. The carbohydrate structure and peptide sequence were deduced from the carbohydrate-related ions and peptide-related ions in the product ion spectra. The structural assignment of the glycopeptide was confirmed by the accurate mass acquired on the FT ICR-MS. The b- and y-ions arising from the peptide backbone in the MS/MS/MS spectra were also used for the peptide assignment. The carbohydrate heterogeneity at each glycosylation site was characterized by integrating the mass spectra of the glycopeptides which yielded identical peptide-related ions. We successfully determined the site-specific glycosylation in LAMP, OBCAM, neurotrimin, and Kilon with the exception of Asn120 in LAMP, Asn113 in OBCAM, Asn120 in neurotrimin, and Asn270 in Kilon. We also demonstrated the structure of the GPI moiety using LC−MS^n equipped with a GCC. A set of data was picked out from all MS data by using GPI-characteristic ions, and the structure of GPI and the linkage site were deduced from the product ions in the MS/MS spectra. Three different structures are commonly found in LAMP, OBCAM, and neurotrimin.

Figure 9 illustrates the site-specific glycosylation in the four proteins. N-Glycosylation sites near the N-terminus in LAMP, OBCAM, and neurotrimin were commonly occupied with biantennary complex-type and hybrid-type oligosaccharides containing disialic acids. Oligosialic acids and disialic acids, which are found in several glycoproteins, including NCAM, are considered to regulate the cell−cell interaction by changing their degree of polymerization (*6*). Disialic acids at the near N-terminus in LAMP, OBCAM, and neurotrimin might regulate the cell−cell interaction in a manner similar to that of other glycosylated adhesion molecules.

The first domains in IgLON family proteins are commonly glycosylated with Man-5, -6, -7, -8, and -9. The linkage of high-mannose-type oligosaccharides is found in several Ig superfamily proteins, including L1, MAG, and P0 (*3*). Since Horstkorte et al. have reported that L1 binds to NCAM through oligomannosidic carbohydrates in L1 (*34*), the high-mannose-type oligosaccharide in IgLON family proteins could interact with certain biological molecules.

The third domains of all IgLON proteins were highly heterogeneous due to a linkage of diverse oligosaccharides, including BA-2, the Le$^{a/x}$ or Le$^{b/y}$ motif, and Man-5. BA-2, a bisected agalacto-complex type, is known as a brain-specific glycan and is much more abundant in mammalian brains than in other tissues (*35, 36*). Recently, the Na^+/K^+-ATPase $\beta1$ subunit was identified as a GlcNAc-binding protein in the mouse brain (*37*). The Na^+/K^+-ATPase $\beta1$ subunit is a potassium-dependent lectin which binds to GlcNAc-terminating oligosaccharides and is involved in neural cell interactions in a trans-binding fashion. A 74 kDa protein was suggested to be the GlcNAc-terminating glycan carrier protein binding to the Na^+/K^+-ATPase $\beta1$ subunit. The linkage of BA-2 to IgLON family proteins implies that these proteins might be the ligand proteins for the Na^+/K^+-ATPase $\beta1$ subunit.

Glycosylation in a great number of membrane glycoproteins remains largely unknown. This is mainly because the limited amount of available sample and the low solubility of glycoproteins make their isolation quite difficult. Our strategy, which includes enrichment of the target glycoproteins, separation by SDS−PAGE, and LC−MS^n of digests of a protein mixture, can be applied to the site-specific glycosylation analysis of various membrane glycoproteins.

ACKNOWLEDGMENT

We thank Dr. Masayuki Kubota and Morihiko Yoshida (Thermo Fisher Scientific K.K.) for their technical support.

REFERENCES

1. Walsh, F. S., and Doherty, P. (1997) Neural cell adhesion molecules of the immunoglobulin superfamily: Role in axon growth and guidance. *Annu. Rev. Cell Dev. Biol. 13*, 425–456.
2. Kleene, R., and Schachner, M. (2004) Glycans and neural cell interactions. *Nat. Rev. Neurosci. 5*, 195–208.
3. Krog, L., and Bock, E. (1992) Glycosylation of neural cell adhesion molecules of the immunoglobulin superfamily. *APMIS, Suppl. 27*, 53–70.
4. Schachner, M., and Martini, R. (1995) Glycans and the modulation of neural-recognition molecule function. *Trends Neurosci. 18*, 183–191.
5. Liedtke, S., Geyer, H., Wuhrer, M., Geyer, R., Frank, G., Gerardy-Schahn, R., Zahringer, U., and Schachner, M. (2001) Characterization of N-glycans from mouse brain neural cell adhesion molecule. *Glycobiology 11*, 373–384.
6. Rutishauser, U. (1996) Polysialic acid and the regulation of cell interactions. *Curr. Opin. Cell Biol. 8*, 679–684.
7. Kunemund, V., Jungalwala, F. B., Fischer, G., Chou, D. K., Keilhauer, G., and Schachner, M. (1988) The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol. 106*, 213–223.
8. Cho, T. M., Hasegawa, J., Ge, B. L., and Loh, H. H. (1986) Purification to apparent homogeneity of a μ-type opioid receptor from rat brain. *Proc. Natl. Acad. Sci. U.S.A. 83*, 4138–4142.
9. Funatsu, N., Miyata, S., Kumanogoh, H., Shigeta, M., Hamada, K., Endo, Y., Sokawa, Y., and Maekawa, S. (1999) Characterization of a novel rat brain glycosylphosphatidylinositol-anchored protein (Kilon), a member of the IgLON cell adhesion molecule family. *J. Biol. Chem. 274*, 8224–8230.
10. Levitt, P. (1984) A monoclonal antibody to limbic system neurons. *Science 223*, 299–301.
11. Pimenta, A. F., Zhukareva, V., Barbe, M. F., Reinoso, B. S., Grimley, C., Henzel, W., Fischer, I., and Levitt, P. (1995) The limbic system-associated membrane protein is an Ig superfamily member that mediates selective neuronal growth and axon targeting. *Neuron 15*, 287–297.
12. Schofield, P. R., McFarland, K. C., Hayflick, J. S., Wilcox, J. N., Cho, T. M., Roy, S., Lee, N. M., Loh, H. H., and Seeburg, P. H. (1989) Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact. *EMBO J. 8*, 489–495.
13. Struyk, A. F., Canoll, P. D., Wolfgang, M. J., Rosen, C. L., D'Eustachio, P., and Salzer, J. L. (1995) Cloning of neurotrimin

defines a new subfamily of differentially expressed neural cell adhesion molecules. *J. Neurosci. 15*, 2141–2156.
14. Brauer, A. U., Savaskan, N. E., Plaschke, M., Prehn, S., Ninnemann, O., and Nitsch, R. (2000) IG-molecule Kilon shows differential expression pattern from LAMP in the developing and adult rat hippocampus. *Hippocampus 10*, 632–644.
15. Gil, O. D., Zhang, L., Chen, S., Ren, Y. Q., Pimenta, A., Zanazzi, G., Hillman, D., Levitt, P., and Salzer, J. L. (2002) Complementary expression and heterophilic interactions between IgLON family members neurotrimin and LAMP. *J. Neurobiol. 51*, 190–204.
16. Hachisuka, A., Nakajima, O., Yamazaki, T., and Sawada, J. (2000) Developmental expression of opioid-binding cell adhesion molecule (OBCAM) in rat brain. *Brain Res. Dev. Brain Res. 122*, 183–191.
17. Miyata, S., Matsumoto, N., Taguchi, K., Akagi, A., Iino, T., Funatsu, N., and Maekawa, S. (2003) Biochemical and ultrastructural analyses of IgLON cell adhesion molecules, Kilon and OBCAM in the rat brain. *Neuroscience 117*, 645–658.
18. Zacco, A., Cooper, V., Chantler, P. D., Fisher-Hyland, S., Horton, H. L., and Levitt, P. (1990) Isolation, biochemical characterization and ultrastructural analysis of the limbic system-associated membrane protein (LAMP), a protein expressed by neurons comprising functional neural circuits. *J. Neurosci. 10*, 73–90.
19. Hachisuka, A., Yamazaki, T., Sawada, J., and Terao, T. (1996) Characterization and tissue distribution of opioid-binding cell adhesion molecule (OBCAM) using monoclonal antibodies. *Neurochem. Int. 28*, 373–379.
20. Wada, Y., Tajiri, M., and Yoshida, S. (2004) Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. *Anal. Chem. 76*, 6560–6565.
21. Wuhrer, M., Hokke, C. H., and Deelder, A. M. (2004) Glycopeptide analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry reveals novel features of horseradish peroxidase glycosylation. *Rapid Commun. Mass Spectrom. 18*, 1741–1748.
22. Satomi, Y., Shimonishi, Y., and Takao, T. (2004) N-Glycosylation at Asn(491) in the Asn-Xaa-Cys motif of human transferrin. *FEBS Lett. 576*, 51–56.
23. Zaia, J. (2004) Mass spectrometry of oligosaccharides. *Mass Spectrom. Rev. 23*, 161–227.
24. Wuhrer, M., Catalina, M. I., Deelder, A. M., and Hokke, C. H. (2007) Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci 849*, 115–128.
25. Wuhrer, M., Koeleman, C. A., Hokke, C. H., and Deelder, A. M. (2005) Protein glycosylation analyzed by normal-phase nano-liquid chromatography-mass spectrometry of glycopeptides. *Anal. Chem. 77*, 886–894.
26. Harazono, A., Kawasaki, N., Kawanishi, T., and Hayakawa, T. (2005) Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS. *Glycobiology 15*, 447–462.
27. Sandra, K., Devreese, B., Van Beeumen, J., Stals, I., and Claeyssens, M. (2004) The Q-Trap mass spectrometer, a novel tool in the study of protein glycosylation. *J. Am. Soc. Mass Spectrom. 15*, 413–423.
28. Itoh, S., Kawasaki, N., Harazono, A., Hashii, N., Matsuishi, Y., Kawanishi, T., and Hayakawa, T. (2005) Characterization of a gel-separated unknown glycoprotein by liquid chromatography/multistage tandem mass spectrometry: Analysis of rat brain Thy-1 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Chromatogr., A 1094*, 105–117.
29. Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem. 256*, 1604–1607.
30. Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R., and Rodriguez-Boulan, E. (1988) Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proc. Natl. Acad. Sci. U.S.A. 85*, 9557–9561.
31. Itoh, S., Kawasaki, N., Ohta, M., and Hayakawa, T. (2002) Structural analysis of a glycoprotein by liquid chromatography-mass spectrometry and liquid chromatography with tandem mass spectrometry. Application to recombinant human thrombomodulin. *J. Chromatogr., A 978*, 141–152.
32. Kikuchi, M., Hatano, N., Yokota, S., Shimozawa, N., Imanaka, T., and Taniguchi, H. (2004) Proteomic analysis of rat liver peroxisome: Presence of peroxisome-specific isozyme of Lon protease. *J. Biol. Chem. 279*, 421–428.
33. Nakajima, O., Hachisuka, A., Takagi, K., Yamazaki, T., Ikebuchi, H., and Sawada, J. (1997) Expression of opioid-binding cell adhesion molecule (OBCAM) and neurotrimin (NTM) in *E. coli* and their reactivity with monoclonal anti-OBCAM antibody. *NeuroReport 8*, 3005–3008.
34. Horstkorte, R., Schachner, M., Magyar, J. P., Vorherr, T., and Schmitz, B. (1993) The fourth immunoglobulin-like domain of NCAM contains a carbohydrate recognition domain for oligomannosidic glycans implicated in association with L1 and neurite outgrowth. *J. Cell Biol. 121*, 1409–1421.
35. Chen, Y. J., Wing, D. R., Guile, G. R., Dwek, R. A., Harvey, D. J., and Zamze, S. (1998) Neutral N-glycans in adult rat brain tissue: Complete characterisation reveals fucosylated hybrid and complex structures. *Eur. J. Biochem. 251*, 691–703.
36. Nakakita, S., Natsuka, S., Ikenaka, K., and Hase, S. (1998) Development-dependent expression of complex-type sugar chains specific to mouse brain. *J. Biochem. 123*, 1164–1168.
37. Kitamura, N., Ikekita, M., Sato, T., Akimoto, Y., Hatanaka, Y., Kawakami, H., Inomata, M., and Furukawa, K. (2005) Mouse Na^+/K^+-ATPase β1-subunit has a K^+-dependent cell adhesion activity for β-GlcNAc-terminating glycans. *Proc. Natl. Acad. Sci. U.S.A. 102*, 2796–2801.

BI8009778

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Biologicals 36 (2008) 27–36

IABS BIOLOGICALS

www.elsevier.com/locate/biologicals

Prion removal by nanofiltration under different experimental conditions

Mikihiro Yunoki [a,b,*], Hiroyuki Tanaka [b], Takeru Urayama [a,b], Shinji Hattori [b], Masahiro Ohtani [b], Yuji Ohkubo [b], Yoshiyasu Kawabata [b], Yuuki Miyatake [b], Ayako Nanjo [b], Eiji Iwao [c], Masanori Morita [b], Elaine Wilson [d], Christine MacLean [d], Kazuyoshi Ikuta [a]

[a] *Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan*
[b] *Research & Development Division, Benesis Corporation, Japan*
[c] *Pharmaceutical Research Division, Mitsubishi Pharma Corporation, Japan*
[d] *BioReliance, Invitrogen BioServices, UK*

Received 21 December 2006; revised 11 April 2007; accepted 27 April 2007

Abstract

Manufacturing processes used in the production of biopharmaceutical or biological products should be evaluated for their ability to remove potential contaminants, including TSE agents. In the present study, we have evaluated scrapie prion protein (PrP^{Sc}) removal in the presence of different starting materials, using virus removal filters of different pore sizes. Following 75 nm filtration, PrP^{Sc} was detected in the filtrate by Western blot (WB) analysis when a "super-sonicated" microsomal fraction derived from hamster adapted scrapie strain 263K (263K MF) was used as the spike material. In contrast, no PrP^{Sc} was detected when an untreated 263K MF was used. By using spike materials prepared in a manner designed to optimize the particle size distribution within the preparation, only 15 nm filtration was shown to remove PrP^{Sc} to below the limits of detection of the WB assays used under all the experimental conditions. However, infectious PrP^{Sc} was recovered following 15 nm filtration under one experimental condition. The results obtained suggest that the nature of the spike preparation is an important factor in evaluating the ability of filters to remove prions, and that procedures designed to minimize the particle size distribution of the prion spike, such as the "super-sonication" or detergent treatments described herein, should be used for the preparation of the spike materials.
© 2007 The International Association for Biologicals. Published by Elsevier Ltd. All rights reserved.

Keywords: Prion; Removal; Filter; Clearance study; Spike material

1. Introduction

The transmission of variant Creutzfeldt–Jakob disease (vCJD) through blood transfusion has been of increasing concern, since a fourth possible transmission case was reported [1]. In addition, prions have been detected in the buffy coat separated from the blood of hamsters infected with scrapie, using a biochemical assay (protein misfolding cyclic amplification, or PMCA) [2]. Infectious prions are thought to be the causative agent of the transmissible spongiform encephalopathy (TSE) diseases, which include Creutzfeldt–Jakob disease (CJD), vCJD, and bovine spongiform encephalopathy (BSE). Therefore, to reduce the risk of transmission when raw materials for protein products (such as plasma) are contaminated with infectious prions, measures should be introduced to decrease the prion load, to evaluate the risk to the product, and to introduce prion removal/inactivation step(s) in the manufacturing process, if feasible [3–5]. Unlike viruses, the minimum infectious prion unit does not exist as a single particle. The infectious prion unit is believed to be composed of protein polymers/aggregates, rather than a prion particle. The unusual nature of the prion agent makes it particularly important to

* Corresponding author. Hirakata Research Laboratory, Research & Development Division, Benesis Corporation, 2-25-1 Shodai-Ohtani, Hirakata, Osaka 573-1153, Japan. Tel.: +81 72 850 0100; fax: +81 72 864 2341.

E-mail address: yunoki.mikihiro@mk.m-pharma.co.jp (M. Yunoki).

1045-1056/07/$34.00 © 2007 The International Association for Biologicals. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.biologicals.2007.04.005