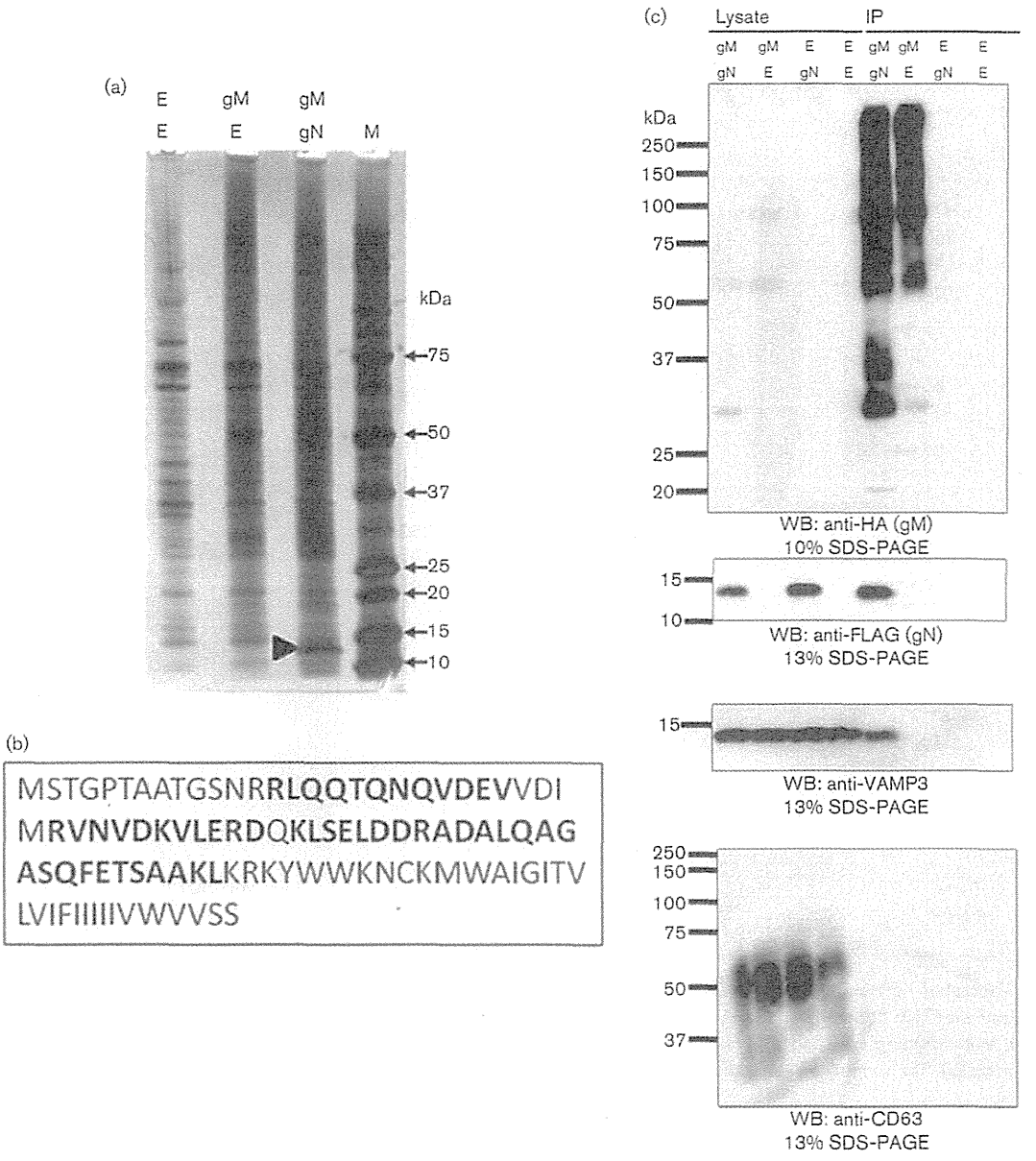


Fig. 1. Interaction between gM/gN and VAMP3. (a) 293T cells were transfected with plasmids expressing HA-tagged gM and FLAG-tagged gN. The cells were lysed with TNE buffer at 72 h post-transfection. The lysates were then immunoprecipitated with an anti-HA Ab specific for gM and visualized by silver staining. The band marked by the arrowhead indicates the protein selected for analysis by LC-MS/MS. (b) Peptide matches to the VAMP3 sequence are shown in bold. (c) 293T cells were transfected with plasmids expressing HA-tagged gM, FLAG-tagged gN, or pCAGGS (negative control). The cells were then lysed with TNE buffer at 72 h post-transfection. The lysates were immunoprecipitated with anti-HA antibody for gM and analysed by Western blotting with anti-HA, anti-FLAG, anti-VAMP3 (BioReagents) or anti-CD63 antibodies. The numbers beside the panels indicate the molecular masses (kDa). WB, Western blotting; E, empty.

VAMP3 at the TGN or a TGN-derived compartment. As expected, when gM was coexpressed with gN, it colocalized with VAMP3 and TGN46 (Fig. 5b); however, this colocalization was not observed when gM was expressed alone [Fig. 5a(ii)]. These results suggest that the interaction between the gM/gN complex and VAMP3 occurs at the TGN or a TGN-derived compartment. Glycoprotein M did not colocalize with CD63 even when gM was coexpressed with gN [Fig. 4c(i)]. Non-specific staining of gM was not seen in these cells [Fig. 5a(iii), b(ii), c(ii)].

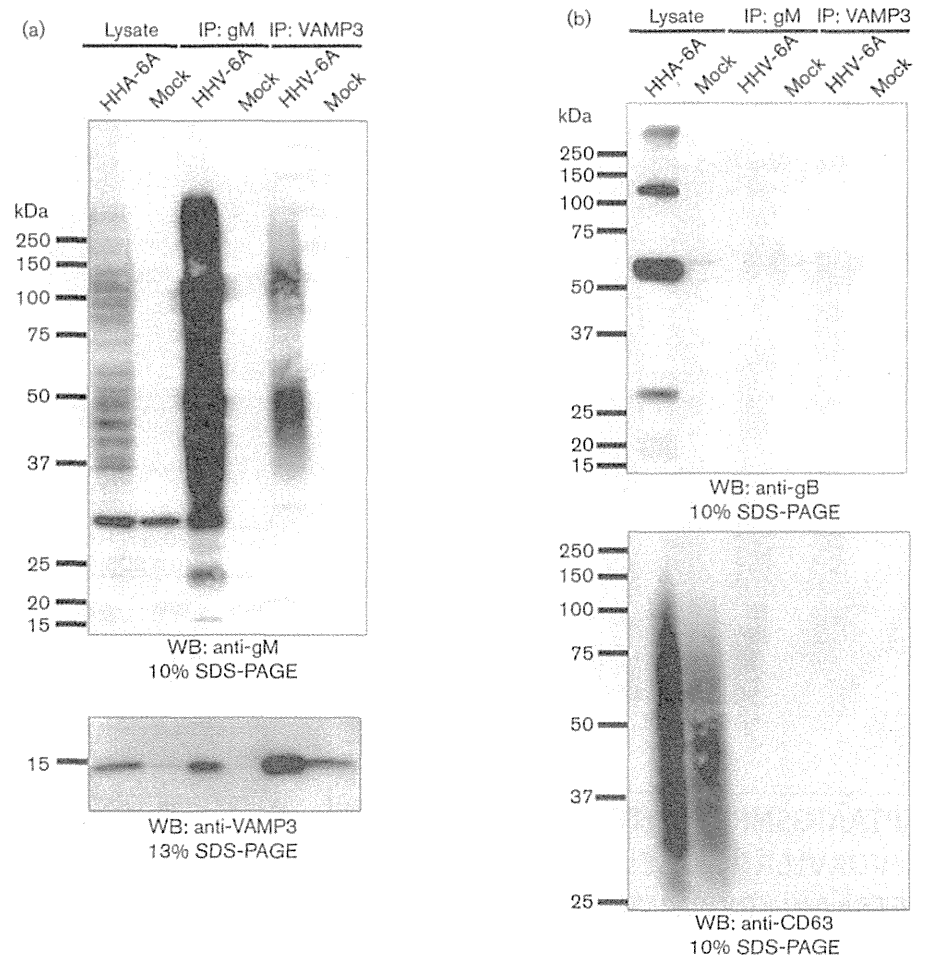


Fig. 2. Interaction between gM and VAMP3 in HHV-6A-infected HSB-2 cells. HHV-6A-infected or mock-infected HSB-2 cells were lysed with TNE buffer at 96 h post-infection. The lysates were immunoprecipitated (IP) with anti-gM mAb or anti-VAMP3 Ab (see Methods) and analysed by Western blotting with anti-gM or anti-VAMP3 (BioReagents) Abs (a), anti-gB Ab or anti-CD63 mAb (b). The numbers beside the panels indicate the molecular masses (kDa). WB, Western blotting.

The kinetics of VAMP3 expression in HHV-6A-infected cells

As shown in Fig. 2, VAMP3 expression in HHV-6A-infected cells was higher than that in mock-infected cells. Therefore, we examined the kinetics of VAMP3 expression in HHV-6A-infected cells. The results in Fig. 6 show that VAMP3 expression increased gradually in the infected cells.

DISCUSSION

Here, we used the transient expression of gM and gN to identify VAMP3 as a cellular molecule that interacts with the HHV-6A gM/gN complex. The interaction between VAMP3 and the gM/gN complex was also confirmed in HHV-6A-infected cells. VAMP3 and gM/gN proteins colocalized at the TGN in cells coexpressing gM and gN, and in HHV-6A-infected cells. This interaction was observed only when gM/gN formed a complex, indicating that the interaction is required for gM/gN complex formation. Previously, we reported that the localization of HHV-6A gM to the TGN was necessary for its interaction with gN (Kawabata *et al.*, 2012). Therefore, the interaction between the gM/gN complex and VAMP3 might also occur at the TGN. It is still not known whether the interaction between VAMP3 and gM requires gM/gN complex formation. Transport of gM to the TGN might be required for this interaction.

VAMP3 also colocalized with CD63, which is a marker of late endosome in HHV-6A-infected cells. In cells transiently expressing gM and gN, however, VAMP3 colocalized with TGN46, but not CD63. This suggests that in infected cells, the localization of VAMP3 may be modified through its interaction with gM/gN, thereby possibly allowing it to localize to the other organelles, such as the late endosome.

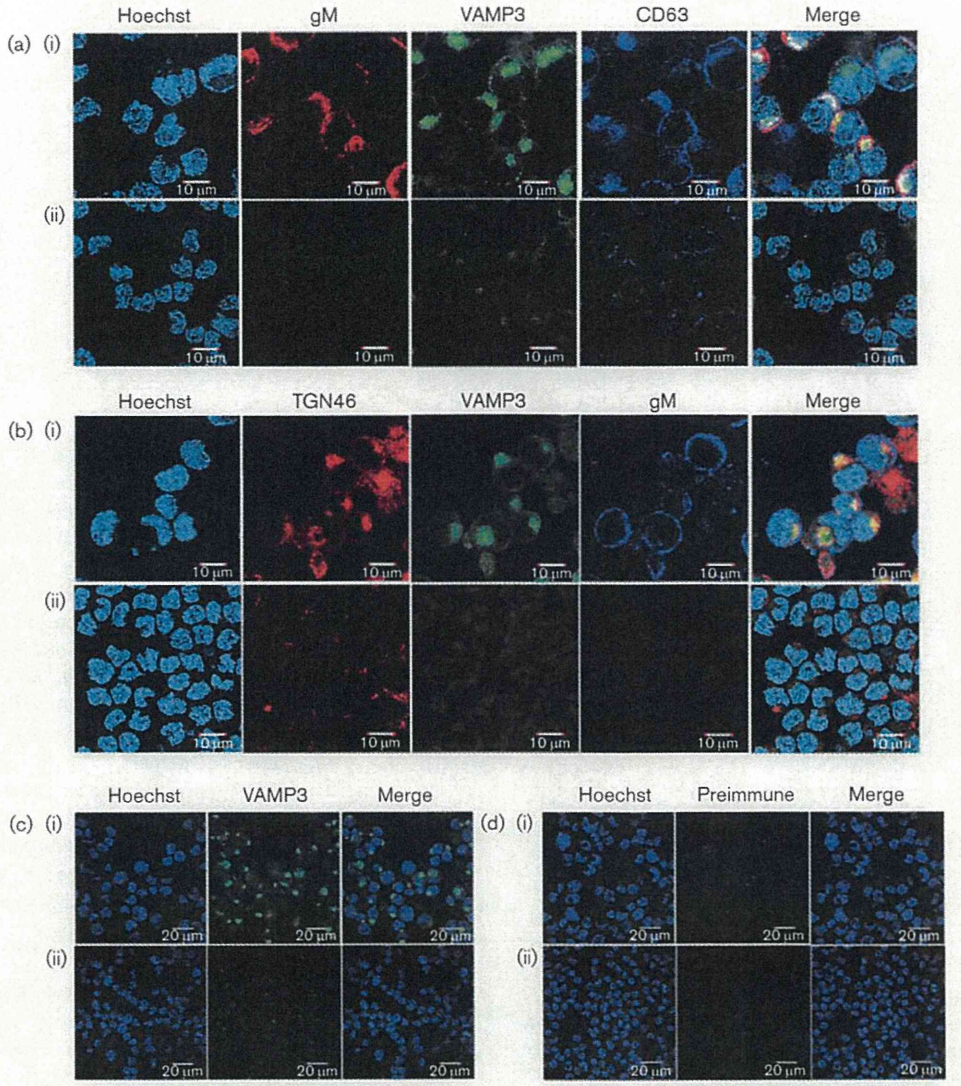


Fig. 3. Colocalization of gM, VAMP3 and CD63, or gM, VAMP3 and TGN46 in HHV-6A-infected cells. HHV-6A-infected [a(i), b(i)] or mock-infected [a(ii), b(ii)] HSB-2 cells were harvested at 96 h post-infection and fixed. The cells were stained with antibodies against VAMP3, gM and CD63 as well as with Hoechst 33258 (a), or VAMP3, gM and TGN46 as well as with Hoechst 33258 (b). Costained areas appear white in the merged panel. Bars, 10 μm. HHV-6A-infected [c(i), d(i)] or mock-infected [c(ii), d(ii)] cells were stained with guinea pig antisera against VAMP3 (c) or preimmune sera obtained from the same guinea pig as well as with Hoechst 33258 (d). Bars, 20 μm.

We also found that VAMP3 was incorporated into virions. As the gM/gN complex is expressed on virions and exosomes, complex-associated VAMP3 would be transported along with the gM/gN complex and then released via the exosomal release pathway (Mori *et al.*, 2008).

Although the function of VAMP3 in HHV-6A-infected cells is not known, its interaction with the gM/gN complex may modify the cellular machinery in infected cells. As VAMP3 is incorporated into virions and exosomes, its primary function (to facilitate membrane fusion) may be lost in infected cells. Overexpression of VAMP3 did not affect HHV-6 growth (data not shown). Several v-SNARE proteins with functions similar to those of VAMP3 have been identified (Borisovska *et al.*, 2005). Therefore, the function of VAMP3 may be redundant in HHV-6A-infected cells. It is still not known whether v-SNAREs, including VAMP3, are required for HHV-6 infection. Further studies will be required to address these questions.

METHODS

Cells and viruses. The HSB-2 T-cell line was cultured in RPMI 1640 medium (Nissui) supplemented with 8% FBS. Human embryonic

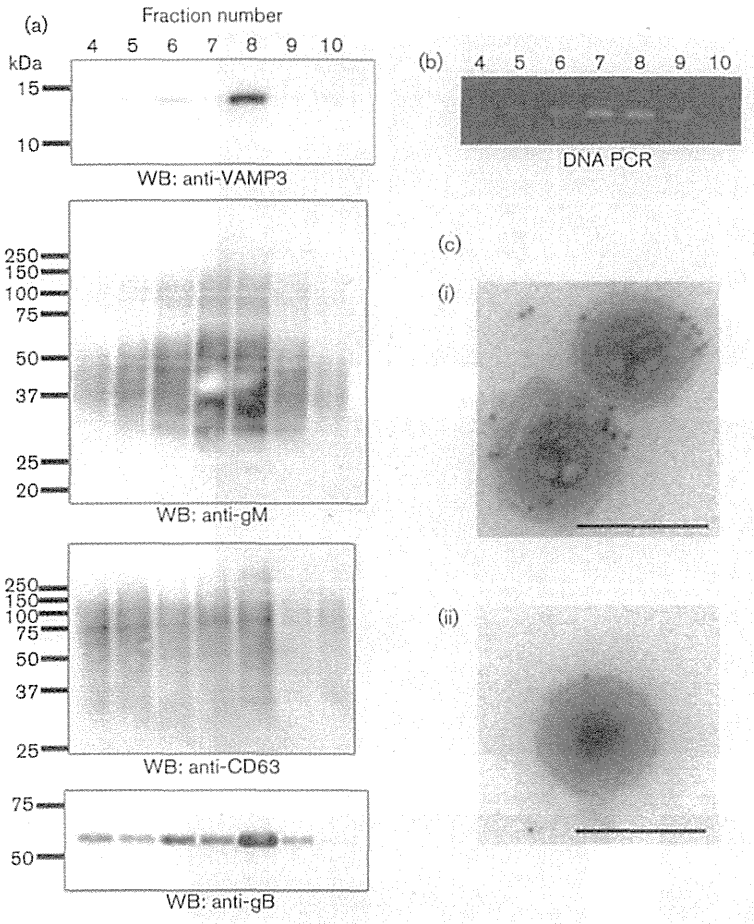


Fig. 4. The presence of VAMP3 in HHV-6A virions and exosomes. Exosome fractions containing virions were purified from the culture medium of HHV-6A-infected cells by sucrose density-gradient centrifugation and then analysed by Western blotting (a), DNA PCR (b) and electron microscopy (c). (a) Western blot analysis of the sucrose density-gradient fractions with anti-VAMP3, anti-gM, anti-CD63 or anti-gB Abs. (b) Viral DNA was detected in the fractions by PCR with HHV-6A specific primers. The numbers above the PCR image showed the fraction numbers. (c) Immunogold localization of VAMP3 on the HHV-6A virions. Purified virions (fraction 8) were labelled with antisera against VAMP3 (i) or without primary antibody (ii). Bars, 200 nm.

kidney cells (293T cells) and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8 % FBS. The HHV-6A strain GS was propagated and titrated in HSB-2 cells. HHV-6A cell-free virus was prepared as previously described (Akkapaiboon *et al.*, 2004). Cord blood mononuclear cells (CBMC) were used for virus propagation (Mori *et al.*, 2004). CBMCs were kindly provided by K. Adachi (Minoh Hospital, Minoh, Japan) and H. Yamada (Kobe University Graduate School of Medicine, Kobe, Japan) and purchased from the RIKEN Cell Bank (BioResource Center). For the usage of CBMCs, the study was approved by the ethics committee of each institution.

Antibodies. Rabbit antibody (Ab) specific for HHV-6A gM or gB (Mori *et al.*, 2008), an AgM-1 mAb against gM (Kawabata *et al.*, 2012), and a U14 mAb against HHV-6 U14 (Takemoto *et al.*, 2005) were used. mAbs against CD63 (clone CLB-gran/12, 435; Sanquin) and α-tubulin (clone B-5-1-2; Sigma), a sheep polyclonal Ab against TGN46 (AbD Serotec), a rabbit polyclonal Ab against VAMP3 (BioReagents) and a goat polyclonal Ab against VAMP3 (Santa Cruz) were used. Anti-HA (clone HA-7; Sigma) and anti-FLAG (clone M2; Sigma) antibodies were also used. Alexa Fluor 594-conjugated donkey anti-sheep IgG (Molecular Probes), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes), FITC-conjugated affinity pure $F(ab')_2$ fragment goat anti-guinea pig IgG (Jackson ImmunoResearch Laboratories), and Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. An anti-VAMP3 monospecific Ab was produced by subjecting guinea pigs to three rounds of immunization with the antigen, which was then expressed in *Escherichia coli* and purified (Mori *et al.*, 2008).

Immunofluorescence assay. The IFA was performed as described previously (Akkapaiboon *et al.*, 2004; Mori *et al.*, 2004). Specific immunofluorescence was observed under a confocal laser-scanning microscope (FluoView FV1000; Olympus).

Plasmid construction. The HA-tagged gM- and FLAG-tagged gN-expressing plasmids were described previously (Kawabata *et al.*, 2012). The pCAGGS plasmid was kindly provided by Jun-ichi Miyazaki (Osaka University, Japan) (Niwa *et al.*, 1991). To express the recombinant protein, the following primer pair was used to amplify inserts from HSB-2 cells cDNA: for named GST-VAMP3, VAMP3FbamHI (5′-ACCGGATCCTCTACAGGTCCAACTGCTGC-CACT-3′) and VAMP3rsalI (5′-ACCGTCGACTTACTTGCAATTCT-TCCACCAATATTTC-3′). The PCR products were inserted into the pGEX-4T1 vector (GE Healthcare).

Plasmid transfection. HeLa cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The 293T cells were transfected using the calcium phosphate method as described previously (Koshizuka *et al.*, 2010).

Identification of gM/gN-interacting proteins. Plasmids expressing HA-tagged gM and FLAG-tagged gN were cotransfected into 293T cells. Cotransfection of gM and pCAGGS into 293T cells was performed as a control. At 72 h post-transfection, the cells were lysed in TNE buffer (0.01 M Tris/HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 % Nonidet-P-40). After centrifugation at 200 000 *g* for 1 h, the

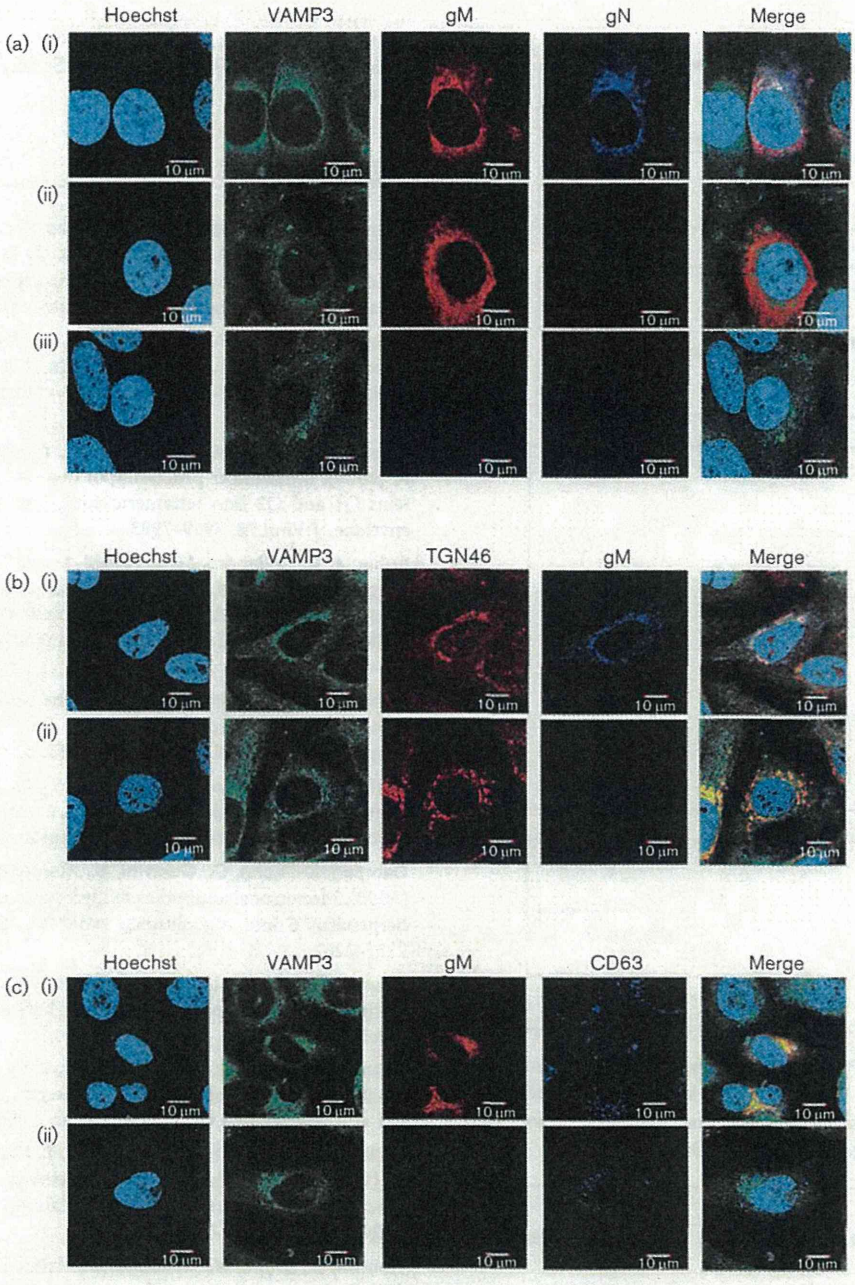


Fig. 5. Subcellular localization of VAMP3 with the gM/gN complex in HeLa cells transiently expressing gM and gN. HeLa cells were transfected with plasmids expressing gM and gN [a(i), b(i), c(i)], gM and the empty vector [a(ii)], or without vectors [a(iii), b(ii), c(ii)]. The cells were harvested at 48 h post-transfection and fixed. (a) The cells were stained with antibodies against gM, FLAG (for gN) and VAMP3 as well as with Hoechst 33258. (b) Cells were stained with antibodies against gM, TGN46 and VAMP3 as well as Hoechst 33258. (c) Cells were stained with antibodies against gM, CD63 and VAMP3 as well as with Hoechst 33258. Co-stained areas appear white or yellow in the merged panel. Bars, 10 μm.

supernatants were incubated overnight at 4 °C with an anti-HA antibody conjugated to protein G Sepharose (GE Healthcare). The protein-anti-HA conjugated beads were then washed with lysis buffer and the bound proteins were eluted with 0.1 M glycine/HCl (pH 2.8). After the beads were removed by centrifugation, the supernatants were neutralized by adding 1 M Tris-HCl (pH 9.5). The eluted proteins were then solubilized with sample buffer, separated on a NuPAGE SDS-PAGE system (Invitrogen), and examined by silver staining. Specific bands were analysed by LC-MS/MS to identify the coimmunoprecipitated proteins (Shevchenko *et al.*, 1996; Tang *et al.*, 2013).

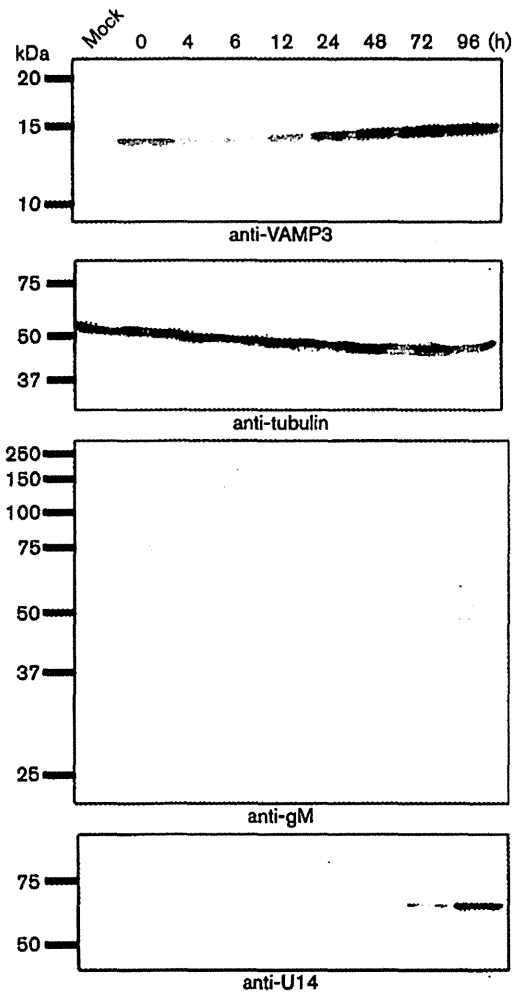


Fig. 6. Kinetics of VAMP3 protein expression in HHV-6A-infected cells. Whole-cell lysates collected at the indicated time points (h) were analysed by Western blotting. The numbers beside the panels indicate the molecular masses (kDa).

Western blotting. Western blotting was performed as described previously (Akkapaiboon *et al.*, 2004).

Isolation of virion fractions. Virions containing exosomes were collected from the cell culture medium by differential centrifugation and fractionated with a linear sucrose gradient, as described previously (Mori *et al.*, 2008). The fractions were analysed by Western blotting, DNA PCR, and electron microscopy.

Electron microscopy. Immunogold labelling of virions was performed as described previously (Mori *et al.*, 2008). The samples were examined under a Hitachi H-7650 electron microscope.

ACKNOWLEDGEMENTS

We thank E. Moriishi (National Institute of Biomedical Innovation), Mayuko Hayashi and Megumi Ota (Kobe University) for technical assistance, J. Miyazaki (Osaka University) for providing reagents, and K. Adachi (Minoh City Hospital) and H. Yamada (Kobe University) for the CBMCs. This work was supported by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (JSPS).

REFERENCES

Ablashi, D. V., Balachandran, N., Josephs, S. F., Hung, C. L., Krueger, G. R., Kramarsky, B., Salahuddin, S. Z. & Gallo, R. C. (1991). Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates. *Virology* **184**, 545–552.

Ablashi, D., Agut, H., Alvarez-Lafuente, R., Clark, D. A., Dewhurst, S., Diluca, D., Flamand, L., Frenkel, N., Gallo, R. & other authors (2014). Classification of HHV-6A and HHV-6B as distinct viruses. *Arch Virol* **159**, 863–870.

Akkapaiboon, P., Mori, Y., Sadaoka, T., Yonemoto, S. & Yamanishi, K. (2004). Intracellular processing of human herpesvirus 6 glycoproteins Q1 and Q2 into tetrameric complexes expressed on the viral envelope. *J Virol* **78**, 7969–7983.

Aubin, J. T., Collandre, H., Candotti, D., Ingrand, D., Rouzioux, C., Burgard, M., Richard, S., Huraux, J. M. & Agut, H. (1991). Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. *J Clin Microbiol* **29**, 367–372.

Baines, J. D. & Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *J Virol* **65**, 938–944.

Borisovska, M., Zhao, Y., Tsytsyura, Y., Glyvuk, N., Takamori, S., Matti, U., Rettig, J., Südhof, T. & Bruns, D. (2005). v-SNAREs control exocytosis of vesicles from priming to fusion. *EMBO J* **24**, 2114–2126.

Campadelli-Fiume, G., Guerrini, S., Xiaoming, L. & Foà-Tomasi, L. (1993). Monoclonal antibodies to glycoprotein B differentiate human herpesvirus 6 into two clusters, variants A and B. *J Gen Virol* **74**, 2257–2262.

Chandran, B., Tirawatnapong, S., Pfeiffer, B. & Ablashi, D. V. (1992). Antigenic relationships among human herpesvirus-6 isolates. *J Med Virol* **37**, 247–254.

Dijkstra, J. M., Visser, N., Mettenleiter, T. C. & Klupp, B. G. (1996). Identification and characterization of pseudorabies virus glycoprotein gM as a nonessential virion component. *J Virol* **70**, 5684–5688.

Galli, T., Chilcote, T., Mundigl, O., Binz, T., Niemann, H. & De Camilli, P. (1994). Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J Cell Biol* **125**, 1015–1024.

Hobom, U., Brune, W., Messerle, M., Hahn, G. & Koszinowski, U. H. (2000). Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. *J Virol* **74**, 7720–7729.

Hu, C., Hardee, D. & Minnear, F. (2007). Membrane fusion by VAMP3 and plasma membrane t-SNAREs. *Exp Cell Res* **313**, 3198–3209.

Jahn, R. & Scheller, R. H. (2006). SNAREs–engines for membrane fusion. *Nat Rev Mol Cell Biol* **7**, 631–643.

Jahn, R., Lang, T. & Südhof, T. C. (2003). Membrane fusion. *Cell* **112**, 519–533.

Kawabata, A., Jasirwan, C., Yamanishi, K. & Mori, Y. (2012). Human herpesvirus 6 glycoprotein M is essential for virus growth and requires glycoprotein N for its maturation. *Virology* **429**, 21–28.

Koshizuka, T., Ota, M., Yamanishi, K. & Mori, Y. (2010). Characterization of varicella-zoster virus-encoded ORF0 gene–comparison of parental and vaccine strains. *Virology* **405**, 280–288.

Krzyzaniak, M. A., Mach, M. & Britt, W. J. (2009). HCMV-encoded glycoprotein M (UL100) interacts with Rab11 effector protein FIP4. *Traffic* **10**, 1439–1457.

Lawrence, G. L., Nicholas, J. & Barrell, B. G. (1995). Human herpesvirus 6 (strain U1102) encodes homologues of the conserved herpesvirus glycoprotein gM and the alphaherpesvirus origin-binding protein. *J Gen Virol* **76**, 147–152.

Luftman, K., Hasan, N., Day, P., Hardee, D. & Hu, C. (2009). Silencing of VAMP3 inhibits cell migration and integrin-mediated adhesion. *Biochem Biophys Res Commun* **380**, 65–70.

McMahon, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R. & Südhof, T. C. (1993). Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* **364**, 346–349.

Mohrmann, R. & Sørensen, J. B. (2012). SNARE requirements en route to exocytosis: from many to few. *J Mol Neurosci* **48**, 387–394.

Mori, Y. (2009). Recent topics related to human herpesvirus 6 cell tropism. *Cell Microbiol* **11**, 1001–1006.

Mori, Y., Akkapaiboon, P., Yonemoto, S., Koike, M., Takemoto, M., Sadaoka, T., Sasamoto, Y., Konishi, S., Uchiyama, Y. & Yamanishi, K. (2004). Discovery of a second form of tripartite complex containing gH-gL of human herpesvirus 6 and observations on CD46. *J Virol* **78**, 4609–4616.

Mori, Y., Koike, M., Moriishi, E., Kawabata, A., Tang, H., Oyaizu, H., Uchiyama, Y. & Yamanishi, K. (2008). Human herpesvirus-6 induces MVB formation, and virus egress occurs by an exosomal release pathway. *Traffic* **9**, 1728–1742.

Niwa, H., Yamamura, K. & Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.

Osterrieder, N., Neubauer, A., Brandmuller, C., Braun, B., Kaaden, O. R. & Baines, J. D. (1996). The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. *J Virol* **70**, 4110–4115.

Polgár, J., Chung, S. H. & Reed, G. L. (2002). Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. *Blood* **100**, 1081–1083.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C., Studdert, M. J. & The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses (1992). The family *Herpesviridae*: an update. *Arch Virol* **123**, 425–449.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55–63.

Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**, 850–858.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. & Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324.

Takemoto, M., Koike, M., Mori, Y., Yonemoto, S., Sasamoto, Y., Kondo, K., Uchiyama, Y. & Yamanishi, K. (2005). Human herpesvirus 6 open reading frame U14 protein and cellular p53 interact with each other and are contained in the virion. *J Virol* **79**, 13037–13046.

Tang, H., Serada, S., Kawabata, A., Ota, M., Hayashi, E., Naka, T., Yamanishi, K. & Mori, Y. (2013). CD134 is a cellular receptor specific for human herpesvirus-6B entry. *Proc Natl Acad Sci U S A* **110**, 9096–9099.

Tayeb, M. A., Skalski, M., Cha, M. C., Kean, M. J., Scaife, M. & Coppolino, M. G. (2005). Inhibition of SNARE-mediated membrane traffic impairs cell migration. *Exp Cell Res* **305**, 63–73.

Tischer, B. K., Schumacher, D., Messerle, M., Wagner, M. & Osterrieder, N. (2002). The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. *J Gen Virol* **83**, 997–1003.

Veale, K. J., Offenhäuser, C., Lei, N., Stanley, A. C., Stow, J. L. & Murray, R. Z. (2011). VAMP3 regulates podosome organisation in macrophages and together with Stx4/SNAP23 mediates adhesion, cell spreading and persistent migration. *Exp Cell Res* **317**, 1817–1829.

Yamagishi, Y., Sadaoka, T., Yoshii, H., Somboonthum, P., Imazawa, T., Nagaike, K., Ozono, K., Yamanishi, K. & Mori, Y. (2008). Varicella-zoster virus glycoprotein M homolog is glycosylated, is expressed on the viral envelope, and functions in virus cell-to-cell spread. *J Virol* **82**, 795–804.

Yamanishi, K., Shiraki, K., Kondo, T., Okuno, T., Takahashi, M., Asano, Y. & Kurata, T. (1988). Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* **331**, 1065–1067.

Journal of proteome research

Article

pubs.acs.org/jpr

Identification of Sialylated Glycoproteins in Doxorubicin-Treated Hepatoma Cells with Glycoproteomic Analyses

Kanako Azuma,[†] Satoshi Serada,[‡] Shinji Takamatsu,[†] Naoko Terao,[†] Shunsaku Takeishi,[§] Yoshihiro Kamada,[†] Tetsuji Naka,[‡] and Eiji Miyoshi*[,†]

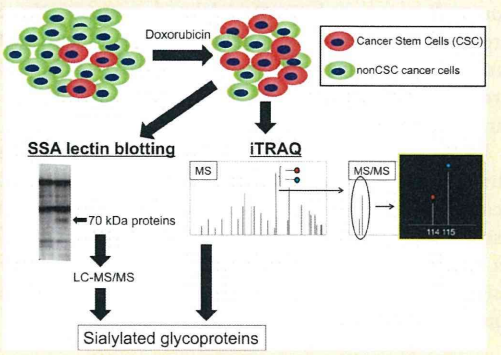
[†]Department of Molecular Biochemistry & Clinical Investigation, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

[‡]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka 567-0085, Japan

[§]Department of Gastroenterology and Oncology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-0855, Japan

Supporting Information

ABSTRACT: Sialylation is one of the most important types of glycosylation involved in carcinogenesis and establishment of cancer stemness. We previously showed that increased sialylation is a characteristic glycan change in cancer stem cells (CSCs) from hepatocellular carcinoma. However, the identities of glycoproteins targeted for sialylation remain unknown. In the present study, we identified glycoproteins targeted for sialylation in doxorubicin (DXR)-treated hepatocarcinoma cell line, Huh7, using glycoproteomic analyses. Since CSCs constitute a small subset of cells within carcinoma cell lines, it is difficult to identify sialylated proteins using general glycoproteomic strategies. It is known that treatment with anticancer drug can condense CSCs, we used DXR to concentrate CSCs. In DXR-treated Huh7 cells, isobaric tag for relative and absolute quantitation (iTRAQ) analysis identified 17 sialylated glycoproteins. Most of the identified glycoproteins were cancer-associated proteins. Furthermore, two proteins of approximately 70 kDa were detected using *Sambucus sieboldoana* agglutinin (SSA) blot analysis and identified as beta-galactosidase and alpha-2-HS-glycoprotein (fetuin-A) by SSA precipitation followed by liquid chromatography-tandem mass spectrometry analyses. Sialylation levels of fetuin-A were increased in DXR-treated Huh7 cell lysates. These changes in sialylation of glycoproteins might be involved in the establishment of cancer stemness.



KEYWORDS: *sialylation, cancer stem cells, doxorubicin, hepatoma, glycoproteomics*

INTRODUCTION

A growing body of evidence suggests that tumors are frequently composed of heterogeneous cell types and that tumor initiation and growth are driven by a small subset of cells, termed cancer stem cells (CSCs), or tumor-initiating cells.[1−3] Several lines of research have indicated that CSCs can be preferentially resistant to many current therapies, including various chemotherapeutic agents and radiation treatment.[4−7] Thus, therapeutic strategies that effectively target CSCs could have a major impact on cancer patient survival. There are many reports on CSC markers, which include CD13,[8] CD44,[9,10] epithelial cell adhesion molecule (EpCAM),[11] and CD133.[9,12,13] These CSC markers have been used for identifying or concentrating CSCs in each type of cancer. It is unknown whether pure CSCs or whether heterogeneous populations containing CSC-like cells, should be targets for therapeutic strategies. Glycans are often attached to proteins and lipids on the cell surface and structurally and functionally modify these molecules. Glycans consist of several kinds of monosaccharides and show great structural diversity. Research in the field of glycobiology has revealed diverse and complex biological roles for these glycans.[14] The structures and amounts of glycans present on the cell surface change dramatically during development and differentiation.[15] We have recently reported that sialylated glycans are useful markers for CSC-like cells in hepatoma cell lines.[16] Glycomic analysis using a lectin microarray showed marked binding of *Sambucus sieboldoana* agglutinin (SSA) to a $CD133^{+}CD13^{+}$ cell subpopulation within hepatoma cell lines. SSA lectin recognizes α2, 6-sialic acid.[16] Sialic acid is one of the building blocks of glycans and is generally found at the outermost ends of the glycan chains of glycoproteins and glycolipids. Thus, sialic acid is associated with many physiological and pathological events, including binding to infectious pathogens, regulation of immune responses, and tumor malignancy.[17] In particular, the alteration of sialic acid moieties is associated with cancer cell behavior, such as invasiveness and metastasis.[18−23] Glycan changes are involved in development and differentiation, and sialic acid is one of the most important glycosylations involved in these processes.[24]

Special Issue: Proteomics of Human Diseases: Pathogenesis, Diagnosis, Prognosis, and Treatment

Received: May 1, 2014
Published: August 26, 2014

In terms of glycoproteomic analyses, the identification of target proteins for each characteristic oligosaccharide structure is very important.[25] However, using the conventional strategy, which involves pooling and concentrating the CSC fraction using CD markers and lectins, it was not easy to obtain appropriate amounts of proteins for glycomic analyses. In general, CSC or CSC-like cells are resistant to anticancer drug treatment. For example, a CD133 and SSA double-positive population was resistant to several kinds of anticancer drug treatments such as 5-fluorouracil (5-FU) (Supplementary Figure 1). Hermann et al. also showed that the number of CD133-positive cells increased in human pancreatic cancer cells treated with gemcitabine.[26] These results suggest that short-term treatment with anticancer drugs can be used to easily concentrate CSCs. Among emerging proteomic technologies, isobaric tags for relative and absolute quantitation (iTRAQ) is a shotgun-based technique, which allows the concurrent identification and relative quantification of hundreds of proteins from different biological samples in a single experiment.[27,28] Whereas iTRAQ analysis leads to a more comprehensive analysis of sialylated proteins, the SSA lectin precipitation technique followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis allows for protein enrichment, which aids in the identification of specific target proteins.

In the present study, we investigated characteristic glycan structures in the doxorubicin (DXR)-treated human hepatoma cell line Huh7, and further identified their target glycoproteins using iTRAQ and SSA lectin precipitation followed by LC–MS/MS. In addition, the expression levels of these proteins were confirmed using Western blotting, and their biological significance in CSC functions is discussed.

■ MATERIALS AND METHODS

Cell Culture and Cell Treatments

The human hepatoma cell line Huh7 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), supplemented with 100 units/mL penicillin G and 100 μg/mL streptomycin in a 37 °C incubator under a humidified atmosphere containing 5% CO_2. The cells were seeded into 100 mm dishes at 1×10^6 cells/dish. After 6 h, doxorubicin (DXR) (Sigma) was added to the culture medium (5 μg/mL). The cells were harvested after 48 h of exposure to DXR, and the culture media were harvested as conditioned media for the following analyses.

Lectin Microarray

Patterns of oligosaccharide structures in Huh7 cells treated with or without DXR were investigated by means of evanescent-field fluorescence-assisted lectin microarray. Forty-five types of lectins were immobilized on a glass slide in triplicate. The procedure has been described in detail, previously.[29] Briefly, total cellular proteins in phosphate buffered saline (PBS) containing 1% Triton X-100 were labeled with Cy3-succinimidyl ester (GE Healthcare, Chalfont St. Giles, U.K.) at room temperature (RT) for 1 h in the dark. Excess reagent was removed by gel filtration chromatography. The resultant Cy3-labeled protein solution was applied to a lectin microarray. After incubation at 20 °C for 15 h, the glass slide was scanned with an evanescent-field fluorescence scanner, GlycoStation (GP Biosciences Ltd., Kanagawa, Japan). All of the data were analyzed using Array Pro Analyzer version 4.5 (Media Cybernetics, Inc., Bethesda, MD, USA). The net intensity value for each spot was calculated by subtracting the background value. The signal intensity value for each lectin was expressed as the average of the net intensity values for three spots. The signal from wheat germ agglutinin (WGA) was used to normalize the signal intensity of each lectin because binding to WGA lectin was relatively stable and similar using different cell types.

Mass Spectrometric Analysis

NanoLC–MS/MS analyses were performed on LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) equipped with nano-ESI source and coupled to Paradigm MG4 pump (Michrom Bioresources, Auburn, CA) and autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). Peptide mixtures were separated on MagicC18AQ column (100 mm × 150 mm, 3.0 μm particle size, 300 Å, Michrom Bioresources) with a flow rate of 500 nL/min. A linear gradient of 5–30% B in 80 min, 30–95% B in 10 min, and 95% B for 4 min and finally decreased to 5% B was employed (A = 0.1% formic acid in 2% acetonitrile; B = 0.1% formic acid in 90% acetonitrile). Intact peptides were detected in the Orbitrap at 30,000 resolution. Up to three CID and HCD spectra were acquired in a data-dependent acquisition mode following each full scan (m/z, 400–1500). The mass spectrometer was operated in positive ion mode.

Preparation of Labeled Peptides for Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) Analysis

Each protein sample (adjusted to 4.1 mg) was digested with 50 μg of Trypsin-TPCK Solution (Applied Biosystems, Framingham, MA, USA) at 37 °C overnight and sialylated proteins were isolated using *Sambucus sieboldiana* agglutinin (SSA) covalently linked to agarose beads (SSA-agarose) (J-Oil Mills, Inc., Tokyo, Japan). After applying to an SSA-agarose column, each sample was incubated for 6 h at 4 °C. The SSA-Agarose column was washed with 1 mL of 50 mM Tris HCl [pH 7.4] buffer five times. Bound peptides were eluted from the SSA-Agarose column using 1 mL of the elution buffer (0.2 M lactose) three times. The eluted samples were deglycosylated with glycopeptidase F. Deglycosylated peptides were desalted and labeled with iTRAQ reagents (Applied Biosystems) according to the manufacturer's instructions. Proteins from Huh7 cells, treated with or without DXR and eluted from SSA agarose, were labeled with iTRAQ reagents 114 and 115, respectively. All labeled peptide samples were mixed and fractionated as described previously.[30]

iTRAQ Data Analysis

Protein identification and quantification for iTRAQ analysis was carried out using Proteome Discoverer v.1.3 (Thermo Fisher Scientific) using the MASCOT algorithm against Swiss-Prot protein database (Swiss-Prot_2012_06 536,489 entries). Taxonomy was set to *Homo sapiens* (20,312 entries). Search parameters for peptide and MS/MS mass tolerance were 10 ppm and 0.8 Da, respectively, with allowance for two missed cleavages made from the trypsin digest. Carbamidomethylation (Cys) and iTRAQ4plex (Lys, N-terminal) were specified as static modifications, whereas deamidation (Asn, Gln), iTRAQ4plex (Tyr), and oxidation (Met) were specified as dynamic modifications in the database search. When deamidation of Asn were detected, the amino acids were considered as glycan binding site because glycopeptidase F treatment convert glycosylated Asn residue to Asp. Mascot results were filtered with the integrated Percolator based filter using a false discovery rate <1% (based on PSMs). Relative protein abundances were calculated using the ratio of iTRAQ reporter ion in the MS/MS scan. List of the

glycoproteins identified in iTRAQ analysis was represented in Supplementary Table 1.

Protein Identification by Mass Spectrometry

The gels were stained with the Silver Stain MS kit according to the manufacturer's instruction (WAKO Pure Chemical Industries, Ltd., Osaka, Japan). Protein spots in a silver-stained gel, corresponding to positive spots on Western blot membranes, were excised from the gel and digested in gel according to a previously described method,[31,32] using sequencing grade modified trypsin (Promega, Inc., Madison, WI). Digested peptides were then extracted with 5% TFA in acetonitrile (acetonitrile/DW 50:45), sonicated for 5 min and concentrated by evaporation. Dried peptides were dissolved in 0.1% TFA (v/v) and 2% acetonitrile (v/v) for subsequent LC–MS/MS analysis. NanoLC–MS/MS analyses were performed on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (AMR) and coupled to a Paradigm MG4 pump (Michrom Bioresources) and an autosampler (HTC PAL, CTC Analytics). A spray voltage of 1800 V was applied. The peptide mixture was separated on a Magic C18AQ column (100 μm × 150 mm, 3.0 μm particle size, 300 Å, Michrom Bioresources) with a flow rate of 500 nL/min. The linear gradient of 5% to 45% B in 30 min, 45% to 95% B in 0.1 min, and 95% B for 2 min and finally decreased to 5% B was employed (A = 0.1% formic acid in 2% acetonitrile; B = 0.1% formic acid in 90% acetonitrile). Intact peptides were detected in the Orbitrap at 60,000 resolutions. For LC–MS/MS analysis, 6 precursor ions were selected for subsequent MS/MS scans in a data-dependent acquisition mode following each full scan (m/z, 350–1500). A lock mass function was used for the LTQ-Orbitrap to obtain constant mass accuracy during gradient analysis. Peptides and proteins were identified by means of automated database search using Proteome Discoverer v.1.3 (Thermo Fisher Scientific) against human of Swiss-Prot protein database (Swiss-Prot_2012_06) with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.8 Da, and strict trypsin specificity, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionines was allowed as dynamic modifications. Raw data of MS/MS analysis was represented in Supplementary Figure 2.

Lectin Blot Analysis

Huh7 cells were quickly harvested from a 100 mm dish in ice-cold PBS. After precipitation by centrifugation at 2000 rpm for 5 min at 4 °C, the cells were resuspended in TNE buffer (10 mM Tris-HCl [pH 7.8], 1% NP40, 1 mM EDTA, and 0.15 M NaCl) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and then placed on ice for 30 min to allow solubilization. Samples were then centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatants were collected as cell lysates. Samples were quantitated using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA).

In each experiment, duplicate samples were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. One gel was subjected to Coomassie Brilliant Blue (CBB) R-250 staining and another was transferred to a polyvinyl difluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA) for lectin blot analysis, using SSA, *Leukoagglutinating phytohemagglutinin* (L4-PHA), *Aleuria aurantia* lectin (AAL), and *Datura stramonium* (DSA). After blocking with PBS containing 3% bovine serum albumin (BSA) overnight at 4 °C, each membrane was incubated in 1:2500–1:10000 diluted biotinylated SSA, L4-PHA, AAL, and DSA (J-Oil Mills, Inc., Tokyo, Japan) for 20 min at RT. The membranes were then washed three times with Tris-buffered saline containing 0.05% Tween-20 (TBST) (pH 7.4) and incubated with 1:2500 diluted avidin–peroxidase conjugates (ABC kits, Vector Res. Corp., Burlingame, CA, USA) for 20 min at RT. The membrane was again washed three times with TBST and then developed using an enhanced chemiluminescence system, Immobilon Western (Millipore), according to the manufacturer's protocol.

Immunoblot Analysis

Cell lysates and conditioned media were subjected to 8% SDS-PAGE under reducing conditions and then transferred to a PVDF membrane (Millipore). After blocking with PBS containing 5% skim milk for 1 h at RT, each membrane was incubated overnight at 4 °C with the following primary antibodies diluted 1:1000–1:5000: anti-HYOU1 (Abnova, Taipei, Taiwan), anti-P4HA1 (Abnova, Taipei City, Taiwan), anti-LAMP1 (Abcam Inc., Cambridge, MA, USA), anti-LAMP2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), antibeta-galactosidase (Santa Cruz Biotechnology), antifetuin-A (Santa Cruz Biotechnology), or anti-β-actin (Cell Signaling, Beverly, MA, USA) for 60 min at RT. The dilution ratios for these antibodies ranged from 1:1000 to 1:5000. The membranes were then washed three times with Tris-buffered saline containing 0.05% Tween-20 (TBST) (pH 7.4) and incubated with 1:5000 diluted horseradish peroxidase conjugated with the appropriate secondary antibody for 30 min at RT. The membranes were again washed three times and developed using an Immobilon Western (Millipore).

Lectin Precipitation

Extracted protein (100 μg) was incubated with 40 μL of SSA-agarose overnight with shaking at 4 °C. Precipitated proteins were washed in 50 mM Tris HCl [pH 7.4] and then boiled in SDS sample buffer. Ten microliters of precipitated proteins were subjected to 8% SDS-PAGE. The gel was stained with Silver Stain MS Kits (Wako, Osaka, Japan). Protein bands were excised from the gel and digested with sequencing grade trypsin (Promega) as described previously.[33]

Neuraminidase/*N*-Glycanase Treatments

The extracted proteins (100 μg) were incubated overnight at 37 °C with 200 mU/mL of neuraminidase (Roche, Nutley, NJ, USA) in 50 mM AcOH buffer [pH 5.5] containing 4 mM $CaCl_2$ and 100 μg/mL BSA. In addition, 100 μg of the extracted proteins were incubated with glycopeptidase F (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. The cell lysates were subsequently boiled in SDS-PAGE sample buffer, electrophoresed on 8% SDS PAGE, and then transferred onto a PVDF membrane (Millipore). After blocking with TBST containing 5% skim milk at 4 °C overnight, Western blot analysis for fetuin-A was performed using antifetuin-A antibody.[34] Briefly, 1:5000 diluted antifetuin-A was incubated in TBST buffer containing 5% skim milk for 1 h at RT. After washing the membrane three times with TBST, it was incubated for 1 h at RT with the secondary antibody, horseradish peroxidase-conjugated antirabbit-IgG (Promega), diluted 1:5000. It was again washed three times and developed with an Immobilon Western (Millipore).

Immunofluorescence Confocal Microscopy

Huh7 cells were seeded in 35 mm dishes at 3×10^5 cells/dish. After 6 h, DXR was added to the culture medium (5 μg/mL). After 48 h of exposure to DXR, cells were washed once in PBS