Extended Core 1 and Core 2 Branched O-Glycans Differentially Modulate Sialyl Lewis x-type L-selectin Ligand Activity*

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Junya Mitoma‡†, Bronislawa Petryniak‡, Nobuyoshi Hiraoka‡†, Jiunn-Chern Yeh‡‡, John B. Lowe‡‡‡, and Minoru Fukuda‡‡ ‡‡
‡‡ From the 3Glycobiology Program, Cancer Research Center, the Burnham Institute, La Jolla, California 92037 and the §Howard Hughes Medical Institute, Department of Pathology, and the Life Sciences Institute, University of Michigan Medical School, Ann Arbor, Michigan 48104

It has been established that sialyl Lewis x in core 2 branched O-glycans serves as an E- and P-selectin ligand. Recently, it was discovered that 6-sulfosialyl Lewis x in core 2 branched O-glycans serves as an E- and P-selectin ligand. This paper is available online at http://www.jbc.org

Mucin-type O-glycans are unique in having clusters of large numbers of O-glycans. These O-glycans contain N-acetylgalactosamine residues at reducing ends, which are linked to serine or threonine residues in a polypeptide (1). These attached O-glycans can be classified into several different groups according to the core structure (2). In many cells, a structure called core 1, Galβ1→3GalNAcα1→3Ser/Thr, functions as an L-selectin ligand in high endothelial venules. Extended core 1 O-glycans can be synthesized when a core 1 extension enzyme is present. In this study, we first show that β1,3-N-acetylgalactosaminyltransferase-3 (β3GlcNAcT-3) is almost exclusively responsible for core 1 extension among seven different β3GlcNAcTs and thus acts on core 1 O-glycans attached to PSGL-1. We found that transcripts encoding β3GlcNAcT-3 were expressed in human neutrophils and lymphocytes but that their levels were lower than those of transcripts encoding core 2 β1,6-N-acetylgalactosaminyltransferase I (Core2GlcNAcT-I). Neutrophils also expressed transcripts encoding fucosyltransferase VII (FucT-VII) and Core2GlcNAcT-I, whereas lymphocytes expressed only small amounts of transcripts encoding FucT-VII. To determine the roles of sialyl Lewis x in extended core 1 O-glycans, Chinese hamster ovary (CHO) cells were stably transfected to express PSGL-1, FucT-VII, and either β3GlcNAcT-3 or Core2GlcNAcT-I. Glycan structural analyses disclosed that PSGL-1 expressed in these transfected cells carried comparable amounts of sialyl Lewis x in extended core 1 and core 2 branched O-glycans. In a rolling assay, CHO cells expressing sialyl Lewis x in extended core 1 O-glycans supported a significant degree of shear-dependent tethering and rolling of neutrophils and lymphocytes, although less than CHO cells expressing sialyl Lewis x in core 2 branched O-glycans. These results indicate that sialyl Lewis x in extended core 1 O-glycans can function as an L-selectin ligand and is potentially involved in neutrophil adhesion on neutrophils bound to activated endothelial cells.

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† Present address: Pathology Div., National Cancer Center Research Inst., 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. ‡‡‡ To whom correspondence should be addressed: The Burnham Inst., 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-646-3144; Fax: 858-646-3193; E-mail: minoru@burnham.org.
‡‡ To whom correspondence should be addressed: The Burnham Inst., 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
§ Investigator of the Howard Hughes Medical Institute. ¶¶‡‡ To whom correspondence should be addressed: The Burnham Inst., 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
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3 The abbreviations used are: Core2GlcNAcT-I, core 2 β1,6-N-acetylgalactosaminyltransferase I; HEV, high endothelial venules; LSST, L-selectin ligand sulfotransferase; FucT-VII, fucosyltransferase VII; β3GlcNAcT, β3-N-acetylgalactosaminyltransferase; CHO, Chinese hamster ovary; RT, reverse transcription; FTTC, fluorescent isothiocyanate; FACS, fluorescence-activated cell sorting; HPLC, high-performance liquid chromatography.

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adhesion to HEV in lymph nodes is only marginally impaired, and MECA-79 antibody staining that decorates HEV is not reduced (17). Recent studies reveal that L-selectin ligand activity remaining after abrogation of Core2GlcNAcT-I is due to the activity of 6-sulfosalicyl Lewis x in extended core 1 O-glycans, NeuNAcα2→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAcβ1→3Galβ1→4GlcNAcα1→Ser/Thr (18). Moreover, a minimum MECA-79 epitope was found to be a 6-sulfo structure in the extended core 1 O-glycan, and MECA-79 antibody binds efficiently to 6-sulfosalicyl Lewis x-containing extended core 1 O-glycans (18). These findings are consistent with previous findings that MECA-79 antibody inhibits lymphocyte adhesion to HEV without removal of sialic acid (19) or fucose and that MECA-79 staining remains after expression of fucose is abrogated by inactivation of fucosyltransferase VII (FucT-VII) (20).

Extended core 1 structure is synthesized by core 1 β1,3-N-acetylglucosaminyltransferase (β3GlcNAcT), which adds β1,3-linked N-acetylglucosamine to Galβ1→3GlcNAcα1→R (Fig. 1). A cDNA encoding β3GlcNAcT was first cloned by expression cloning, and the encoded protein was designated β1-antigen forming β1,3-N-acetylglucosaminyltransferase (β1GlcNAcT) and β1,3-galactosyltransferase IV (β1GalT-IV) (54). This is followed by sialylation and fucosylation by α2,3-sialyltransferase and FucT-VII, forming sialyl Lewis x in core 2 branch (left). Core 1 is also modified by core 1 β3GlcNAcT, which is also known as β3GlcNAcT-3, forming extended core 1. Extended core 1 is then galactosylated (most likely by β1,4-galactosyltransferase I), sialylated, and fucosylated, forming sialyl Lewis x in extended core 1 structure (middle). Core 1 can be sialylated by β-galactosidase α2,3-sialyltransferase I (ST3Gal I) (50) and then by N-acetylgalactosamine α2,6-sialyltransferase (ST6GalNac), forming disialosyl core 1 O-glycan (right). This biosynthetic pathway precludes either core 1 extension or core 2 branching.
Expression of α Antigen in HeLa Cells by Different β3GlcNAcTs—To determine whether all of β3GlcNAcTs direct the synthesis of poly-N-acetyllactosamine, HeLa cells were transiently transfected with one of the pcDNA3.1(N)-β3GlcNAcTs or pcDNA1.1-β3GlcNAcT-3. Thirty-six hours after transfection, cells were dissociated into monodispersed cells using an enzyme-free cell dissociation solution (Hanks’ balanced saline solution-based) purchased from Cell and Molecular Technologies. Monodispersed cells were incubated with human anti-i antibody, followed by affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM antibodies (Fierce). The stained cells were subjected to FACS analysis using a FACSScan (BD Biosciences) as described previously (32).

HeLa cells were chosen as recipient cells for transfection because the molecular structure of the membrane-bound glycoprotein-1 containing minimum amounts of poly-N-acetyllactosamine (33). Expression of MECA-79 in Lec2 Cells by LSST and a β3GlcNAcT—To determine which β3GlcNAcT directs expression of MECA-79 antigen, Lec2 cells were transiently transfected with pcDNA1.1-β3GlcNAcT-3. Thirty-six hours after transfection, cells were dissociated into monodispersed cells using the cell dissociation solution as described above. Monodispersed cells were incubated with MECA-79 antibody (BD Biosciences) (19), followed by affinity-purified FITC-conjugated goat anti-rat IgM antibody (ICN Biochemicals). The stained cells were subjected to FACS analysis as described above. CHO mutant Lec2 cells lack a functional Golgi CMP-sialic acid transporter; therefore, sialylation is absent in Lec2 cells (34). The absence of sialylation facilitates core 1 extension because core 1 extension and sialylation compete with each other for the same acceptor, Galβ1→3GalNAcα1→R.

Core 1 Extension in PSGL-1 by β3GlcNAcT—To determine which β3GlcNAcT can add β1,3-N-acetylgalactosamine to core 1, Galβ1→3Galα1→R, Lec2 cells were transiently transfected with pcDNA1.1-β3GlcNAcT and pZeoSV-PSGL-1 (kindly provided by Dr. Richard Cummings) and vectors encoding β3GlcNAcT using LipofectAMINE as described previously (12). The ratio of pZeoSV-PSGL-1 and β3GlcNAcT cDNA was 1.5 (w/w) to achieve efficient modification of PSGL-1 by β3GlcNAcT. Forty-eight hours after transfection, cells were harvested in phosphate-buffered saline with a cell scraper. The cells were subjected to SDS-PAGE. After blotting onto a polyvinylidene difluoride membrane filter, the blot was reacted with anti-PSGL-1 antibody (KPL-1, BD Biosciences), followed by secondary antibody; and immunoreactive PSGL-1 was visualized using enhanced Luminol reagent (PerkinElmer Life Sciences).

RT-PCR of β3GlcNAcT-3, Core2GlcNAcT-I, LSST, Fuc-T VII, and PSGL-1 on RNA Isolated from Neutrophils and Lymphocytes—Human neutrophils and lymphocytes were isolated after hypotonic lysis of erythrocytes. Total RNA was extracted to SDS-PAGE. After blotting onto a polyvinylidene difluoride membrane filter, the blot was reacted with anti-PSGL-1 antibody (KPL-1, BD Biosciences), followed by secondary antibody; and immunoreactive PSGL-1 was visualized using enhanced Luminol reagent (PerkinElmer Life Sciences).

Isolation of Oligosaccharides Attached to PSGL-1 IgG Chimeric Protein from Transfected CHO Cells—RNA encoding PSGL-1/IGG chimeric protein was constructed using pZeoSV-PSGL-1 and pSV-hygromycin as described previously (12), resulting in pcDNA1.1-PSGL-1/IGG. CHO-PSGL-1 cells were then transiently transfected with pC9DMS-Fuc-T VII and pSV-hygromycin and cultured in the presence of 100 μg/ml Geneticin (Invitrogen). CHO-PSGL-1/C1 cells stably expressing Fuc-T VII were selected after staining with anti-sialyl Lewis x antibody (CSLEX-1), resulting in CHO-PSGL-1/F7/C1 cells.

As a control, CHO-PSGL-1 cells were stably transfected with pcDNA1.1-Core2GlcNAcT-I together with pcDNA3 and cultured in the presence of 100 μg/ml Zeocin and 400 μg/ml Genetin. Cells expressing Core2GlcNAcT-I were chosen for expressing larger forms of PSGL-1 that contains core 2 branched O-glycans. Expression of core 2 O-glycans was confirmed after transient transfection of CD43 (also called leukosialin) and staining with T905 antibody, resulting in CHO-PSGL-1/C2 cells. CHO-PSGL-1/C2 cells were then transiently transfected with pC9DMS-Fuc-T VII and pSV-hygromycin and cultured in the presence of 100 μg/ml Zeocin, hygromycin B, and Geneticin. Cells expressing Fuc-T VII were selected after staining with CSLEX-1 antibody, resulting in CHO-PSGL-1/F7/C2 cells.

Stable Expression of β3GlcNAcT-3 and Core2GlcNAcT-I in CHO Cells—CHO cells were first transfected with pZeoSV-PSGL-1 and selected in the presence of 100 μg/ml Zeocin (Invitrogen). CHO colonies stably expressing PSGL-1 were selected after staining with anti-PSGL-1 antibody KPL-1, establishing CHO-PSGL-1 cells. CHO-PSGL-1 cells were stably cotransfected with pcDNA1.1-β3GlcNAcT-3 (core 1 β3GlcNAcT) and pSV-hygromycin and selected in 100 μg/ml Zeocin and 400 μg/ml hygromycin B (Calbiochem). Expression of β3GlcNAcT-3 was shown by the expression of larger forms of PSGL-1 that contains extended core 1 O-glycans (see also “Results”). The resultant CHO-PSGL-1 cells were stably transfected with pC9DMS-Fuc-T VII and pSV-hygromycin and cultured in the presence of 100 μg/ml Geneticin (Invitrogen). CHO-PSGL-1/C1 cells stably expressing Fuc-T VII were selected after staining with anti-sialyl Lewis x antibody (CSLEX-1), resulting in CHO-PSGL-1/F7/C1 cells.

In parallel, CHO-PSGL-1 cells were transfected with pcDNA1.1-Core2GlcNAcT-I together with pcDNA3 and cultured in the presence of 100 μg/ml Zeocin and 400 μg/ml Genetin. Cells expressing Core2GlcNAcT-I were chosen for expressing larger forms of PSGL-1 that contains core 2 branched O-glycans. Expression of core 2 O-glycans was confirmed after transient transfection of CD43 (also called leukosialin) and staining with T905 antibody, resulting in CHO-PSGL-1/C2 cells. CHO-PSGL-1/C2 cells were then transiently transfected with pC9DMS-Fuc-T VII and pSV-hygromycin and cultured in the presence of 100 μg/ml Zeocin, hygromycin B, and Geneticin. Cells expressing Fuc-T VII were selected after staining with CSLEX-1 antibody, resulting in CHO-PSGL-1/F7/C2 cells.

As a control, CHO-PSGL-1 cells were stably transfected with pC9DMS-Fuc-T VII and pcDNA3 and cultured in the presence of Zeocin and Geneticin. Cells expressing Fuc-T VII were selected after staining with anti-PSGL-1 antibody (KPL-1, BD Biosciences), followed by secondary antibody; and immunoreactive PSGL-1 was visualized using enhanced Luminol reagent (PerkinElmer Life Sciences).

RT-PCR of β3GlcNAcT-3, Core2GlcNAcT-I, LSST, Fuc-T VII, and PSGL-1 on RNA Isolated from Neutrophils and Lymphocytes—Human neutrophils and lymphocytes were isolated after hypotonic lysis of erythrocytes. Total RNA was extracted and subjected to SDS-PAGE. After blotting onto a polyvinylidene difluoride membrane filter, the blot was reacted with anti-PSGL-1 antibody (KPL-1, BD Biosciences), followed by secondary antibody; and immunoreactive PSGL-1 was visualized using enhanced Luminol reagent (PerkinElmer Life Sciences).

Isolation of Oligosaccharides Attached to PSGL-1 IgG Chimeric Protein from Transfected CHO Cells—RNA encoding PSGL-1/IGG chimeric protein was constructed using pZeoSV-PSGL-1 and pSV-hygromycin, obtaining PSGL-1/F7/C2 cells. As reported previously, T305 reacts with core 2 branched O-glycans. Expression of i antigen in CHO cells was analyzed using enhanced Luminol reagent (PerkinElmer Life Sciences).

Structural Analysis of Oligosaccharides Attached to PSGL-1 IgG Chimeric Protein from Transfected CHO Cells—RNA encoding PSGL-1/IGG chimeric protein was constructed using pZeoSV-PSGL-1 and pSV-hygromycin, obtaining PSGL-1/F7/C2 cells. As reported previously, T305 reacts with core 2 branched O-glycans. Expression of i antigen in CHO cells was analyzed using enhanced Luminol reagent (PerkinElmer Life Sciences).
nitrile, 45% H$_2$O, and 5% 0.25 M KH$_2$PO$_4$/H$_2$O) over the next 50 min. The sample was finally eluted with 100% of solution B over the last 30 min.

Standard O-glycans were obtained from CD34/IgG glycans synthesized in the presence of β3GlcNAcT-3 and Core2GlcNAcT-I as described previously (12, 18). Oligosaccharides were digested with Streptomyces sp. α1,3/4-fucosidase (Panvera/Takara, Madison, WI) and jack bean β-galactosidase (Sigma) as described previously (39, 41, 42). The digest was desalted by Sephadex G-25 gel filtration in 7% 1-propanol before HPLC analysis.

**Measurement of L-selectin-mediated Rolling in CHO Cells Expressing Sialyl Lewis x in Extended Core 1 or Core 2 Branched O-Glycans—CHO cells stably expressing PSGL-1, FucT-VII, and β3GlcNAcT-I were established as described above. These cells maintained similar amounts of sialyl Lewis x and PSGL-1 as assessed by FACS analysis using anti-i antibody, followed by FITC-conjugated antibody, and subjected to FACS (thick solid lines). Control experiments were performed by transfection with pcDNA3.1(N) without a cDNA insert, followed by staining with (thin solid lines) or without (broken line in Vector panel) anti-i antibody.

Because Galβ1→4(sulfo→6)GlcNAcβ1→3Galβ1→3GalNAcα1→R is a minimum epitope for MECA-79 antigen, the formation of extended core 1 can be detected by immunostaining with MECA-79 antibody when LSST is also expressed.

After the initial report on core 1 β3GlcNAcT (or β3GlcNAcT-3) (18), three additional β3GlcNAcTs highly related to β3GlcNAcT-3 were molecularly cloned: β3GlcNAcT-5 (25, 26), β3GlcNAcT-6 (27), and β3GlcNAcT-7 (28). We thus determined whether extended core 1 can be formed by these more recently identified members of the β3GlcNAcT gene family.

First, we tested whether all of the cloned β3GlcNAcTs are active in synthesizing i antigen, Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc→R (21, 31). The synthesis of i antigen is dependent on β3GlcNAcT-I, which adds β1,3-linked GlcNAc to N-acetyllactosamine. HeLa cells were thus transiently transfected with one of the β3GlcNAcTs in mammalian expression vectors, and the transfected cells were stained with anti-i antibody, followed by FITC-conjugated secondary antibody. Fig. 2 shows that the cells transfected with any of the β3GlcNAcTs tested displayed increased amounts of i antigen compared with mock-transfected cells. The results also indicate that the expression efficiency of different β3GlcNAcTs is essentially invariable because similar amounts of i antigen were detected in HeLa cells transfected with different β3GlcNAcTs.

As shown previously, CHO cells and the CHO mutant Lec2 cell line do not synthesize core 2 O-glycans (43) or extended core 1 oligosaccharides (18). Lec2 cells were used as a reporter cell line to test β3GlcNAcT-dependent reconstitution of MECA-79 antigen expression because Lec2 cells are deficient in core 1 extension and thus MECA-79 antigen expression and because the sialylation defect in Lec2 cells will not inhibit...
Because all of the antigen, but none of the other enzymes formed MECA-79 antigen. Western blot analysis using anti-PSGL-1 monoclonal antibody KPL-1. The transfected Lec2 cells were then subjected to polymerase chain reaction (PCR) products derived from neutrophils (N) and lymphocytes (L) were digested with the indicated restriction enzymes and separated by electrophoresis on 2% agarose gel. Control reactions employed total RNA that was not reverse-transcribed (−). Control reactions employed total RNA that was not reverse-transcribed (−). The products were separated by electrophoresis on 1% agarose gels. Transcripts encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as a positive control. To determine whether \( \beta \)-GalNAcT-3 is also expressed in human neutrophils and lymphocytes, although the amount of the transcript was much less than that of the Core2GalNAcT-I transcript. In addition, the level of Core2GalNAcT-I transcripts was less than that of PSGL-1. Formulation of MECA-79 antigen by sialylation of \( \beta \)-GalNAcTs. These results indicate that neutrophils express each of the enzymes necessary to form sialyl Lewis x in extended core 1 structure, but its product is smaller than that formed by \( \beta \)-GalNAcT-3 or \( \beta \)-GalNAcT-2. These results indicate that \( \beta \)-GalNAcT-3 is almost exclusively responsible for extending core 1.

FIG. 4. Extension of core 1 O-glycans attached to PSGL-1 by different \( \beta \)GalNAcTs. Lec2 cells were transiently transfected with vectors encoding PSGL-1 and one of the \( \beta \)GlcNAcTs. PSGL-1 in the membrane fraction was separated by SDS-PAGE, followed by Western blot analysis using anti-PSGL-1 monoclonal antibody KPL-1 and secondary antibody. Control experiments (−) were obtained by transfection with a cDNA insert-free vector. PSGL-1 migrated as a monomer and a dimer.

FIG. 5. Detection of \( \beta \)GlcNAcT-3, Core2GlcNAcT-I, FucT-VII, LSST, and PSGL-1 transcripts in human neutrophils and lymphocytes. A, cDNAs were synthesized by reverse transcriptase using total RNA isolated from human neutrophils and lymphocytes. PCR was carried out using the cDNAs as templates and oligonucleotide primers specific to each transcript (+). Control reactions employed total RNA that was not reverse-transcribed (−). The products were separated by electrophoresis on 1% agarose gels. Transcripts encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as a positive control. B, PCR products derived from neutrophils (N) and lymphocytes (L) were digested with the indicated restriction enzymes and separated by electrophoresis on 2% agarose gel. Positive controls employed PCR using plasmids (P) containing respective cDNAs as templates. The migration positions of molecular mass markers are shown on the left.

migrated at \( \sim 125 \) kDa. By contrast, \( \beta \)GlcNAcT-3 converted PSGL-1 into polydisperse higher molecular mass glycoforms of \( \sim 150–200 \) kDa. Apparently, \( \beta \)GlcNAcT-2 can form similar products with very low efficiency, and \( \beta \)GlcNAcT-6 forms small amounts of extended core 1, but its product is smaller than that formed by \( \beta \)GlcNAcT-3 or \( \beta \)GlcNAcT-2. These results indicate that \( \beta \)GlcNAcT-3 is almost exclusively responsible for extending core 1. Formulation of MECA-79 antigen by sialylation of Galβ1→3GalNAcOac−→R. Lec2 cells were thus transiently transfected with LSST and one of the \( \beta \)GlcNAcTs. As shown in Fig. 3, Lec2 cells transfected with \( \beta \)GlcNAcT-3, also called core 1-\( \beta \)GlcNAcT, expressed significant amounts of MECA-79 antigen, but none of the other enzymes formed MECA-79 antigen. Because all of the \( \beta \)GlcNAcTs tested were expressed in similar amounts in HeLa cells, it is reasonable to assume that these \( \beta \)GlcNAcTs were expressed in similar amounts in Lec2 cells as well (see also Fig. 4). These results indicate that only \( \beta \)GlcNAcT-3 can form extended core 1 structure.

Modification of PSGL-1 O-Glycans by \( \beta \)GlcNAcTs—To confirm the above conclusions, Lec2 cells were then subjected to Western blot analysis using anti-PSGL-1 antibody KPL-1.

Fig. 4 illustrates that PSGL-1 in mock-transfected Lec2 cells migrated at \( \sim 125 \) kDa. By contrast, \( \beta \)GlcNAcT-3 converted PSGL-1 into polydisperse higher molecular mass glycoforms of \( \sim 150–200 \) kDa. Apparently, \( \beta \)GlcNAcT-2 can form similar products with very low efficiency, and \( \beta \)GlcNAcT-6 forms small amounts of extended core 1, but its product is smaller than that formed by \( \beta \)GlcNAcT-3 or \( \beta \)GlcNAcT-2. These results indicate that \( \beta \)GlcNAcT-3 is almost exclusively responsible for extending core 1.

Core 1 Extension Enzyme (\( \beta \)GlcNAcT-3) Is Also Expressed in Neutrophils and Lymphocytes—Previously, we showed that \( \beta \)GlcNAcT-3 is expressed in HEV in peripheral lymph nodes and forms L-selectin ligand critical for lymphocyte homing (18). To determine whether \( \beta \)GlcNAcT-3 is also expressed in human neutrophils and lymphocytes, neutrophils and lymphocytes were isolated from the peripheral blood, and RT-PCR was used to assay for \( \beta \)GlcNAcT-3 transcripts. The results shown in Fig. 5 demonstrate that the \( \beta \)GlcNAcT-3 transcript was expressed in neutrophils and lymphocytes, although the amount of the transcript was much less than that of the Core2GlcNAcT-I transcript. In addition, the level of Core2GlcNAcT-I transcripts was less than that of PSGL-1. Notably, neutrophils, but not lymphocytes, contained a significant amount of FucT-VII transcripts. To confirm that these transcripts are derived from the proper corresponding GlcNAcT locus, the RT-PCR products were digested with restriction enzymes and analyzed by agarose gel electrophoresis. The results shown in Fig. 5d demonstrate that the transcripts from neutrophils and lymphocytes produced the same restriction digest products as those derived from plasmids encoding those proteins, supporting our conclusion that the transcripts derived from these cells represent \( \beta \)GlcNAcT-3, Core2GlcNAcT-I, FucT-VII, and PSGL-1, respectively.

On the other hand, LSST transcripts were barely detected in neutrophils or lymphocytes, indicating that LSST is expressed, if at all, in very low quantities in these cells (Fig. 5). These results indicate that neutrophils express each of the enzymes necessary to form sialyl Lewis x in extended core 1 structure,
whereas lymphocytes express trace amounts of the sialyl Lewis x moiety due to negligible expression of FucT-VII.

**Structural Analysis of PSGL-1 O-Glycans Synthesized in the Presence of β3GlcNAcT-3 or Core2GlcNAcT-I**—Because PSGL-1 is expressed in neutrophils and lymphocytes as indicated, the above results suggest that β3GlcNAcT-3 can form extended core 1 structure in PSGL-1, a counter-receptor for L-, P-, and E-selectins in neutrophils. Such expression led to the formation of sialyl Lewis x structure in extended core 1 glycans when FucT-VII was also present. Fig. 6 illustrates that either β3GlcNAcT-3 (C1) or Core2GlcNAcT-I (C2) could convert PSGL-1/IgG chimeric protein into a polydisperse high molecular mass glycoform, which could be metabolically labeled with [3H]glucosamine (second and third lanes). By contrast, PSGL-1 chimeric protein migrated as sharper bands when isolated from control CHO cells expressing only FucT-VII (first lane).

These samples were treated with alkaline borohydride to release O-glycans and subjected to Sephadex G-50 gel filtration. Released O-glycans (Fig. 7, A and E, *horizontal bars*) were subjected then to Bio-Gel P-4 gel filtration. As shown in Fig. 7 (B and F), PSGL-1 O-glycans derived from CHO cells expressing FucT-VII and β3GlcNAcT-3 or Core2GlcNAcT-I mainly produced two or three peaks (I, I’, and II). After desialylation and Bio-Gel P-4 gel filtration, peak I produced peaks IA and IB (Fig. 7, C and G). As shown in Fig. 7 (C and G), peak C2-IA from Core2GlcNAcT-I-expressing CHO cells eluted slightly later than peak C1-IA from β3GlcNAcT-3-expressing CHO cells.

Peaks C1-IA and C2-IA were then analyzed by HPLC (Fig. 8). Both peaks C1-IA and C2-IA produced two peaks. The first peaks C1-IA1 and C2-IA1 eluted corresponding to Galβ1→4GlcnAcβ1→3Galβ1→3GalNAcOH and Galβ1→4GlcnAcβ1→6Galβ1→3GalNAcOH, respectively. After α1,3-specific fucosidase digestion, the second peaks C1-IA2 and C2-IA2 were converted to peaks C1-IA1 and C2-IA1, respectively (Fig. 8, B and D), indicating that peaks C1-IA2 and C2-IA2 were derived from NeuNacα2→3Galβ1→4(Fucα1→3)GlcNacβ1→3Galβ1→3GalNAcOH and NeuNacα2→3Galβ1→4(Fucα1→3)GlcNacβ1→3Galβ1→3GalNAcOH and NeuNacα2→3Galβ1→4(Fucα1→3)GlcNacβ1→3Galβ1→3GalNAcOH, respectively.

After desialylation, peaks C1-I and C2-I (Fig. 7, B and F) also produced peaks C1-IB and C2-IB, which eluted at the same positions as Galβ1→3GalNAcOH and sialic acid monomer (Fig. 7, C and G). These results indicate that peaks C1-I and C2-I
A branched glycans can be fucosylated more efficiently than core 2 branched structure (Fig. 7, B versus F). These combined results indicate that non-sulfated sialyl Lewis x in extended core 1 O-glycans functions as a more efficient L-selectin ligand, although sialyl Lewis x in core 2 branched O-glycans as a L-selectin ligand, although sialyl Lewis x in extended core 1 O-glycans is expressed at levels equivalent to sialyl Lewis x in extended core 1 O-glycans (Fig. 9, compare A and C; and Table I). However, the conversion of core 1 structure to extended core 1 is less efficient than the conversion of core 1 structure to core 2 branched structure (Fig. 7, B versus F, compare peaks I and II; and Table I). These results as a whole indicate that sialyl Lewis x in core 2 branched O-glycans is expressed at levels equivalent to sialyl Lewis x in extended core 1 O-glycans (Table I).

**DISCUSSION**

In this study, we have demonstrated that β3GlcNAcT-3, formerly called core 1 β3GlcNAcT, is almost exclusively responsible for adding β1,3-linked GlcNAc to Galβ1→3GalNAcα1→R, forming extended core 1 oligosaccharide. Among other β3GlcNAcTs, β3GlcNAcT-2 may add β1,3-linked GlcNAc to core 1 O-glycans with low efficiency, whereas β3GlcNAcT-6 apparently forms small amounts of extended core 1 structure.
β3GlcNAcT-2 is the most efficient enzyme to add N-acetyllactosamine repeats (22), whereas β3GlcNAcT-6 acts on GalNAcα1→Ser/Thr (27). These results suggest that β3GlcNAcT-2 and β3GlcNAcT-6 may act as core 1 extension enzymes with very low efficiency under certain conditions. Indeed, the amino acid sequence of β3GlcNAcT-3 is highly related to that of β3GlcNAcT-6 (51.6% identity), which acts on GalNAcα1→R.

This study unexpectedly demonstrated that extended core 1 is most likely present in neutrophils and lymphocytes, although the amount of extended core 1 structure is less than that of core 2 branched structure. Interestingly, LSST, which is required to form 6-sulfoglucosylation, is apparently not expressed in neutrophils and lymphocytes. This finding is consistent with the fact that neutrophils and lymphocytes are negative for MECA-79 antigen. β3GlcNAcT-3 belongs to the β3GlcNAcTs gene family, which consists of at least eight different β3GlcNAcTs. The members of this gene family include β3GlcNAcTs encoded by fringe and brainiac (44–47). Fringe was identified as a protein that regulates Notch signaling by adding β1,3-linked GlcNAc to α-fucose attached to the extracellular domain of Notch (44, 45). By contrast, Brainiac apparently acts on glycolipids and thereby modulates Notch activity by another mechanism (46, 47). Although β3GlcNAcT-1 does not have discernible homology to the β3GlcNAcT gene family, recent studies show that one protein predicted by DNA sequence within the human or mouse Large locus has some homology to β3GlcNAcT-1 (48). Premature translation termination of this putative glycosyltransferase within the Large locus in mice results in myodystrophy (48), whereas the LARGE locus is deleted in human patients with meningioma, a tumor of the meninges of the central nervous system (49).

These results suggest that β3GlcNAcT-1 may play an important role in development and cancer because β3GlcNAcT-1 is ubiquitously expressed (21).

This study demonstrated that, in transfected CHO cells, fucosylation of N-acetyllactosamine in extended core 1 structure takes place more efficiently than does fucosylation in core 2 branched O-glycans. This finding is consistent with the previous finding that a significant portion of extended core 1 structure is fucosylated in HEV (18). Our results show that extended core 1 structure is efficiently fucosylated once core 1 structure is formed. On the other hand, the amount of extended core 1 O-glycans is less than that of core 2 branched O-glycans (Table 1). As an aggregate, extended core 1 and core 2 branched O-glycans contain similar amounts of sialyl Lewis x. Synthesis of both extended core 1 and core 2 branches competes with α2,3-sialylation of core 1, which is catalyzed by β-galactosidase α2,3-sialyltransferase I (50). It is thus possible that both the levels of expressed β3GlcNAcT-3 and its catalytic activity are not as high as those of expressed Core2GlcNAcT-I in transfected CHO cells. It is also possible that localization of β3GlcNAcT-3, Core2GlcNAcT-I, and β-galactosidase α2,3-sialyltransferase I in different Golgi compartments is a key factor in determining the amount of oligosaccharides synthesized by β3GlcNAcT-3 or Core2GlcNAcT-I (51, 52). Previously, we have shown that Core2GlcNAcT-I resides in the cis- to medial-Golgi, whereas the majority of N- and O-glycan sialyltransf erase s are thought to reside in the medial- to trans-Golgi (51). This difference allows Core2GlcNAcT-I to add core 2 branch before core 1 oligosaccharide is sialylated. If core 1 oligosaccharide is sialylated first, it becomes unavailable for Core2GlcNAcT-I action (see Fig. 1 in Ref. 2). It is tempting to speculate that β3GlcNAcT-3 resides in later compartments of the Golgi than does Core2GlcNAcT-I, thus directly competing with β-galactosidase α2,3-sialyltransferase I for the same acceptor, Galβ1→3GalNAcα1→Ser/Thr. Such direct competition, if it occurs, should lead to moderate synthesis of extended core 1 structure.

Previously, we showed that 6-sulfosialyl Lewis x in extended core 1 O-glycans serves as an L-selectin ligand as efficiently as 6-sulfosialyl Lewis x in core 2 branched O-glycans (18). In this study, we extended this finding by showing that sialyl Lewis x in extended core 1 O-glycans also functions as an L-selectin ligand, although it is not as efficient as sialyl Lewis x in core 2 branched O-glycans. Our results are also consistent with previous reports showing that sialyl Lewis x functions as an L-selectin ligand, although extended core 1 structure was not evaluated in that study (53). In our previous study, we found that the sialyl Lewis x structure is present in extended core 1 O-glycans of HEV-derived GlyCAM-1, which were converted to neutral oligosaccharides after desialylation (18). These results combined indicate that sialyl Lewis x in extended core 1 serves as an L-selectin ligand in HEV. L-selectin-mediated neutrophil rolling was shown to take place in adherent neutrophils bound to activated endothelial cells (10, 11). Very recently, we obtained mice heterozygous for β3GlcNAcT-3 deficiency and knock-in of green fluorescent protein under the control of the β3GlcNAcT-3 promoter. Analysis of these mice indicated that β3GlcNAcT-3 is expressed in neutrophils and T lymphocytes because neutrophils and T lymphocytes stained with markers CD11b (Mac-1) and CD3, respectively, were also positive for green fluorescent protein as determined by FACS analysis. These studies did not, however, inform at how much sialyl Lewis x in extended core 1 structure is present in neutrophils. Further studies on the knockout mice are important to determine the degree to which sialyl Lewis x moieties in extended core 1 structure contribute to L-selectin-mediated adhesion in HEV and neutrophil-neutrophil interaction.

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