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Sialyl Le^x Structures In *O*-Glycans Attached To Lysosomal Membrane Glycoproteins, Lamp-1 And Lamp-2. Comparison to Those in *N*-Glycans.

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Abstract: Poly-*N*-acetyllactosamine extension has been found in *O*-glycans in addition to *N*-glycans and glycosphingolipids (Fukuda, M. (1994) In *Molecular Glycobiology*, (ed. M. Fukuda and O. Hindsgaul), pp. 1-52, Oxford University Press, Oxford). However, the extent of poly-*N*-acetyllactosamine formation between *O*-glycans and *N*-glycans in a given glycoprotein has not been determined. Attempts were made in HL-60 cells to determine the amount of sialyl Le^X structures in *O*-glycans and *N*-glycans attached to the major lysosomal membrane glycoproteins, lamp-1 and lamp-2. Lamp molecules were immunoprecipitated from ^{3}H-glucosamine labeled HL-60 cells. Glycopeptides were prepared by pronase or trypsin digestion, and *O*-glycan containing glycopeptides were isolated by affinity chromatography using Jacalin-Agarose. The glycopeptides bound to Jacalin-Agarose were treated with alkaline borohydride, and the released *O*-glycans were fractionated by Bio-Gel P-4 filtration. Similarly, the glycopeptides unbound to Jacalin-Agarose, which represent *N*-glycans, were isolated by Sephadex G-50 gel filtration. Sequential glycosidase digestion of the *O*- and *N*-glycans, with or without pretreatment by fucosidase or neuraminidase, revealed the following features: (1) each lamp-1 and lamp-2 molecule contains about 2 and 1 poly-*N*-acetyllactosaminyl *O*-glycan chains, (2) about 65% of these poly-*N*-acetyllactosaminyl *O*-glycans contain sialyl Le^X termini, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R, and (3) *N*-glycans and *O*-glycans are modified in almost equal efficiency to express sialyl Le^X structures. These results indicate that in lamp molecules both *O*-glycans and *N*-glycans contribute to the expression of sialyl Le^X structures.

Polylactosaminylglycans are high molecular weight carbohydrates bound to proteins and lipids. Polylactosaminoglycans are unique in having *N*-acetyllactosaminyl repeats $(Gal\beta 1\rightarrow 4GlcNAc\beta 1\rightarrow 3)_n$ and this repeating structure can be identified by its susceptibility to endo-β-galactosidase. Polylactosaminoglycans present in *N*-glycans and glycolipids have been shown to carry various antigenic determinants such as I/i and ABO blood group antigens (for review, see Refs. 1-3). The structures of polylactosaminoglycans can be characteristic to different cell types and stages of differentiation. In particular, it has been demonstrated that human granulocytes and monocytes express Le^X structure, Galβ1→4(Fucα1→3)GlcNAcβ1→R and sialyl Le^X structure, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R at the termini of polylactosaminoglycans.[4,5] These unique structures were found to serve as ligands for adhesive molecules, selectins, in endothelial cells and platelets,[6-9] and interaction between selectins and cell surface carbohydrates of granulocytes and monocytes are thought to play critical roles in the recruitment of leukocytes to inflammatory sites.[10,11]

Although poly-*N*-acetyllactosamines present in *N*-glycans and glycolipids have been extensively studied, it is known that *O*-glycans can also be modified by poly-*N*-acetyllactosamine extension.[12,13] We have shown previously that some of poly-*N*-acetyllactosaminyl *O*-glycans isolated from granulocytes can contain sialyl Le^x structures.[12] It was also found that poly-*N*-acetyllactosamine is almost exclusively extended from the branch attached to C-6 of *N*-acetylgalactosamine.[12,13] However, it is not known as yet which proteins have *O*-glycans containing poly-*N*-acetyllactosamines in these cells, nor how many sialyl Le^x structures are present in *O*-glycans compared to those on *N*-glycans.

We have isolated two distinct groups of major sialylglycoproteins in leukocytes. Leukosialin contains approximately 80 *O*-glycans and one *N*-glycan per molecule.[14] It has been shown that the carbohydrate structures of these *O*-glycans are characteristic of cell lineage and different stages of differentiation.[15,16] We have shown that leukosialin contained, on average, one molecule of sialyl Le^x terminal structure when it was isolated from HL-60 cells.[17] The second group consists of lysosomal membrane glycoproteins, lamp-1 and lamp-2, which contain 18 and 16 *N*-glycans, respectively, and a significant portion of these carry poly-*N*-acetyllactosamines.[18,19] Moreover, we found that lamp-1 and lamp-2 contain 5 and 8 *O*-glycans per molecule, respectively.[20]

These results prompted us to investigate the structures of *O*-glycans attached to lamp molecules, focusing on the structures of poly-*N*-acetyllactosaminyl *O*-glycans and the presence of sialyl Le^x terminal structures.

EXPERIMENTAL PROCEDURES

Metabolic Labeling of HL-60 and K562 Cells - For metabolic labeling of HL-60 cells, the cells were cultured in a mixture of 98% glucose free RPMI 1640 supplemented with 10% dialyzed fetal calf serum and 2% of regular RPMI 1640 medium containing 10% fetal calf serum (final glucose concentration, 0.28 mM). The medium was supplemented with sodium pyruvate (0.11 mg/ml), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). The cells were cultured for 24 h in the presence of [^{3}H] glucosamine or [^{3}H] galactose [DuPont - New England Nuclear] at a concentration of 20 μCi/ml.

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis - Radiolabeled cells were lysed in PBS (6.7 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.002% NaN_3) containing 1% Nonidet P-40 and protease inhibitors as described.[19] Lamp-2 was immunoprecipitated from the cell lysates by the addition of rabbit anti-human lamp-2 antibodies followed by the addition of formalin-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) as described.[19] The supernatant from this immunoprecipitation was then subjected to immunoprecipitation using anti-human lamp-1 antibodies.[20] Aliquots of radiolabeled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 8% polyacrylamide gel according to Laemmli,[21] followed by fluorography using Enlightning (DuPont - New England Nuclear) (Fig. 1).

Lectin Affinity Chromatography of Glycopeptides - Glycopeptides were released by Pronase digestion of immunoprecipitates and fractionated by serial lectin affinity chromatography as described[19] except for Jacalin-Agarose chromatography. Briefly, the glycopeptides were first applied to a column of concanavalin A-Sepharose, and highly branched complex-type *N*-glycan glycopeptides and *O*-glycan containing glycopeptides (Fraction I) were separated from biantennary asparagine-linked glycopeptides (Fraction II) and high-mannose glycopeptides (Fraction III). Fraction I was then applied to *Datura stramonium* agglutinin (DSA)-Agarose. The glycopeptides bound to this column would contain *N*-glycans with poly-*N*-acetyllactosamine repeats and/or R→GlcNAcβ1→6 (R→GlcNAcβ1→2)Man→ branchings (Fraction I B). The glycopeptides unbound to this column were further applied to a column (0.7 x 10.5 cm) of Jacalin-Agarose (Vector Laboratories, Burlingame,

CA). The column was equilibrated with 100 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl,[22] and the glycopeptides dissolved in the same buffer were applied to the column. After washing with the same buffer, the glycopeptides bound to Jacalin-Agarose were eluted with 0.1 M melibiose in the same buffer (Aldrich).[17,22] In order to ensure the complete binding of glycopeptides, the flow rate was maintained at 4 ml/h throughout the procedure. These glycopeptides were desalted by gel filtration on a column of Sephadex G-25 eluted with water containing 7% propanol.

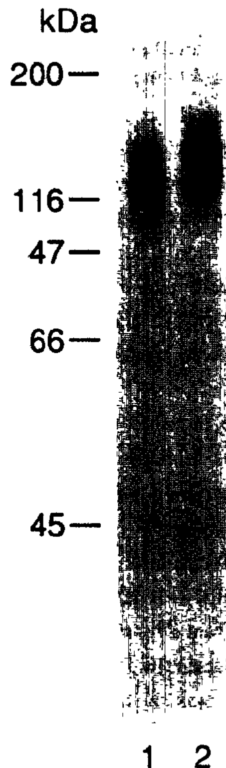

Fig. 1 Sodium dodecylsulfate-polyacrylamide gel electrophoresis of lamp-1 and lamp-2 from HL-60 cells. HL-60 cells were labeled with [^{3}H] glucosamine and lamp-1 and lamp-2 were sequentially immunoprecipitated (lanes 1 and 2). They were then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis followed by fluorography. The molecular weights of standard proteins migrated are indicated at the left.

In order to obtain the whole hinge-region as one glycopeptide, the immunoprecipitates of lamp-1 and lamp-2 were eluted by boiling in 0.1 % SDS for 5 min. The supernatants obtained after brief centrifugation were subjected to reduction with dithiothreitol followed by alkylation with monoiodoacetic acid.[20] To the reduced and alkylated sample was then added 3 volumes of acetone, and the suspension was briefly centrifuged. The precipitates, dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0, were digested at 37°C with 80 μg

of trypsin for lamp-1 or 40 µg of trypsin followed by 4 µg of V8 protease for lamp-2.[20] Total incubation time was 28 h for lamp-1 or 44 h for lamp-2. The digests were applied to a short column of Sephadex G-25 for desalting before being subjected to serial lectin affinity chromatography.

Alkaline Borohydride Treatment of Glycopeptides - In order to release *O*-glycans from glycopeptides, the glycopeptides were incubated in 0.05 M NaOH - 1 M $NaBH_4$ at 45°C for 48 h. After this treatment, the sample was treated with methanol containing 1 M acetic acid and methylborate was removed under a flow of nitrogen in a water bath at 37°C. After repeating this treatment one more time, methanol was added to the sample and then removed as described above. The dried sample was either immediately subjected to Sephadex G-15 gel filtration for desaltion or frozen at -20°C until desaltion.

Fractionation of *O*-glycans and *N*-glycans by Gel Filtration - *O*-Glycans were fractionated by gel filtration using a column (1.0 x 105 cm) of Bio-Gel P-4 (200 to 400 mesh) equilibrated with 0.1 M NH_4HCO_3. *N*-glycans were fractionated by gel filtration using a column (1.0 x 105 cm) of Sephadex G-50 (Superfine) equilibrated with 0.1 M NH_4HCO_3. Fractions of 1 ml were collected at a flow rate of 5 ml/h.

Glycosidase Treatment - Radiolabeled *O*-glycans were digested with 90 munits of endo-β-galactosidase in 100 µl of 50 mM sodium acetate buffer, pH 5.8 under the conditions described previously.[23] Endo-β-galactosidase was purified from *Escherichia freundii*[23] and kindly donated by Dr. Michiko N. Fukuda of our institute. Radiolabeled glycopeptides or *O*-glycans were digested with 40 munits of Newcastle disease virus neuraminidase in 80 µl of 50 mM cacodylate buffer pH6.5 at 37°C for 24 h.[17] It has been shown that Newcastle disease virus neuraminidase can cleave the NeuNAcα2→3 linkage but barely cleaves other linkages or not at all.[24]

The glycopeptides and *O*-glycans were digested with a mixture of 15 munits of diplococcal β-galactosidase and 20 munits of β-*N*-acetylglucosaminidase in 110 µl of 50 mM cacodylate buffer, pH 6.0. If necessary, 50 mM of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Boehringer-Mannheim) was added as an inhibitor for potentially contaminating neuraminidases.[25] If necessary, 100 mM of L-fucose (Sigma) was also added to inhibit potentially contaminating α-L-fucosidase. As shown previously, diplococcal β-galactosidase can cleave Galβ1→4GlcNAc linkage but not Galβ1→3GlcNAc linkage.[26] Glycopeptides and *O*-glycans were digested with 20 munits of α1→3/4 fucosidase from *Streptomyces sp.* 142,[27] together with the same amount of diplococcal β-galactosidase and β-*N*-acetylglucosaminidase as described above, in 90 µl of 25 mM citrate phosphate buffer, pH 6.0. This α-L-fucosidase was shown to cleave Galβ1→4(Fuc α1→3)GlcNAcβ1→R and Galβ1→3(Fuc α1→3)GlcNAcβ1→R but not NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R or NeuNAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→R under these conditions.[17,27] All these digestions were carried out at 37°C for 48 h. Newcastle disease virus neuraminidase was prepared in our laboratory whereas diplococcal β-galactosidase and β-*N*-acetylglucosaminidase were purchased from Boehringer-Mannheim. *Streptomyces* α1→3/4 fucosidase was purchased from Takara (Berkeley, CA). Standard oligosaccharides for *O*-glycans and digestion products after endo-β-galactosidase treatment were obtained from leukosialin and lamp-1 and lamp-2 as described previously.[19,28,29]

RESULTS

Isolation of *O*-Glycans from Lamp-1 and Lamp-2 - Lamp-1 and lamp-2 are extremely enriched with *N*-glycans and less than 10% of the total carbohydrate is due to *O*-glycans. In order to isolate selectively *O*-glycans, glycopeptides were subjected to serial lectin affinity chromatography as shown in Fig. 2. In this

chromatography, the glycopeptides containing *O*-glycans are not bound to ConA-Sepharose nor DSA-Agarose, but should bind to Jacalin-Agarose.

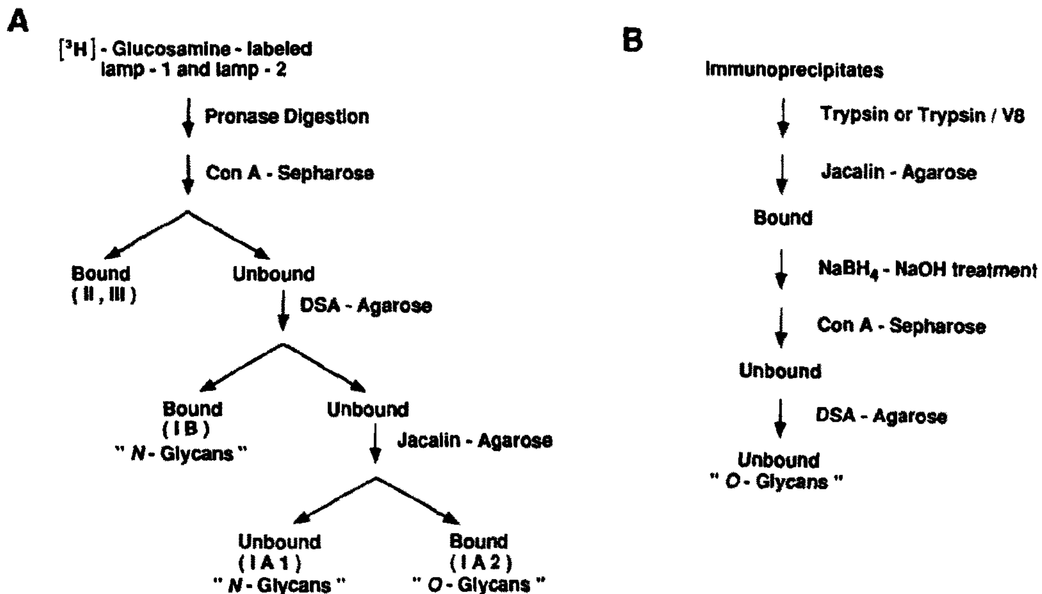

Fig. 2 Experimental procedures used for the isolation of glycopeptides containing *N*-glycans and *O*-glycans.
A) The samples shown in Fig. 1 were digested by Pronase and the radio-labeled glycopeptides were sequentially applied to ConA-Sepharose, DSA-Agarose, followed by Jacalin-Agarose. Fraction IB and Fraction IA1 are glycopeptides containing *N*-glycans whereas Fraction IA2 contain glycopeptides containing *O*-glycans. B) The samples shown in Fig. 1 were digested by trypsin (for lamp-1) or trypsin followed by V8 protease (for lamp-2) and the radiolabeled glycopeptides were applied to a column of Jacalin-Agarose. The fraction bound and eluted from Jacalin-Ararose are glycopeptides containing *O*-glycans. Since this fraction was found to contain contaminating *N*-glycans, they were removed by ConA-Sepharose followed by DSA-Agarose after alkaline borohydride treatment. This fraction, after ConA- and DSA-column purification, still contains a small amount of DSA-unbound *N*-glycans in addition to *O*-glycans (see the text).

First, glycopeptides were prepared by pronase digestion of [^{3}H]-glucosamine labeled lamp-1 and lamp-2 of HL-60 cells and subjected to serial lectin affinity chromatography as shown in Fig. 2A. The glycopeptides bound to Jacalin-Agarose (5-6% of the total radioactivity) were then treated with alkaline borohydride, and the released *O*-glycans were subjected to Bio-Gel P-4 gel filtration. As shown in Fig. 3A and B, those *O*-glycans were separated into 8 fractions, and the structures of oligosaccharides at fractions 3 to 9 were determined as follows.

Oligosaccharide 3 produced the tetrasaccharide, Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH and two sialic acid residues after treatment with Newcastle disease virus neuraminidase, indicating that oligosaccharide 3 is disialosylhexasaccharide, NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcOH. As a minor component (less than 10%) of the total radioactivity, a radioactive peak corresponding to

Galβ1→3[Galβ1→4(Fucα1→3)GlcNAcβ1→6]GalNAcOH was produced, indicating the presence of NeuNAcα2→3Galβ1→3[NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→6]GalNAcOH. Oligosaccharide 5 yielded one sialic acid and the tetrasaccharide, after treatment with Newcastle disease virus neuraminidase. Further treatment with β-galactosidase and β-*N*-acetylglucosaminidase yielded Galβ1→3GalNAcOH, indicating oligosaccharide 5 being monosialosyl pentasaccharide. In order to determine the position of sialic acid substitution in galactose residues, oligosaccharide 5 from lamp-1 (Fig. 3C) was digested with a mixture of β-galactosidase and β-*N*-acetylglucosaminidase from jack bean. After this treatment, 48% of the radioactivity was converted to GlcNAc and monosialosyltrisaccharide. However, 52% of radioactivity remained intact. These results indicate that oligosaccharide 5 is a mixture of NeuNAcα2→3Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH and Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAcOH in a ratio of 48:52. Similarly, the structures of oligosaccharides 6-9 were determined as shown in Table I.

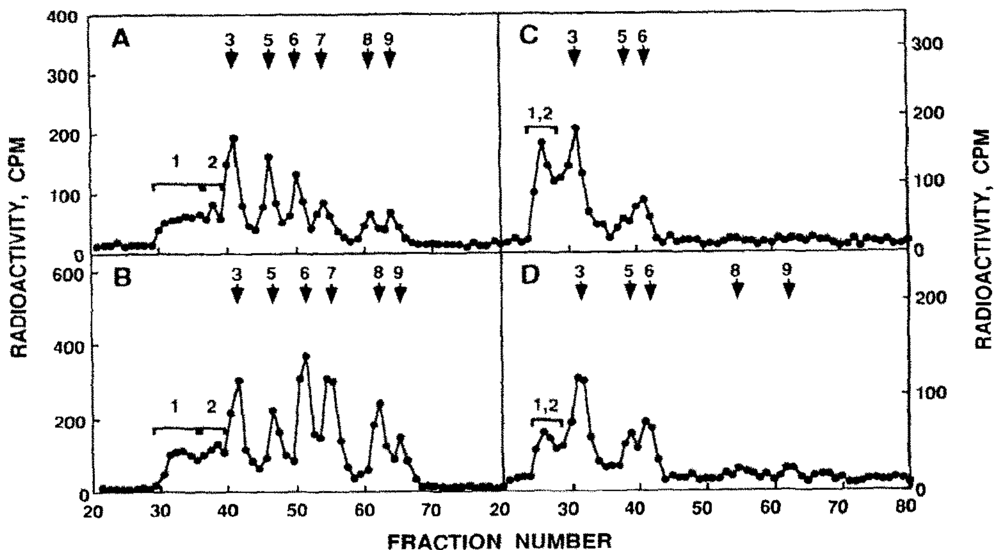

Fig. 3 Bio-Gel P-4 gel filtration of *O*-glycans released from glycopeptides bound to Jacalin-Agarose.
The lamp-1 and lamp-2 glycopeptides bound to Jacalin-Agarose were treated with alkaline borohydride and subjected to Bio-Gel P-4 gel filtration. A, B, *O*-glycans released from Pronase digested glycopeptides of lamp-1 (A) and lamp-2 (B). C, D, *O*-glycans released from trypsin digested lamp-1 glycopeptides (C) and from trypsin-V8 protease digested lamp-2 glycopeptides (D). Peak 1 and peak 2 represent *O*-glycans containing poly-*N*-acetyllactosamine in A and B. In addition, DSA-unbound *N*-glycans are present in peaks 1 and 2 of C and D. The elution positions of standard oligosaccharides are as follows: 3, NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6) GalNAcOH; 5, NeuNAcα2→3Galβ1→3 (Galβ1→4GlcNAcβ1→6) GalNAcOH plus Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6) GalNAcOH; 6, NeuNAcα2→3Galβ1→3GalNAcOH; 7, Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH;
8, Galβ1→3GalNAcOH; 9, GalNAcOH.

TABLE I

Structure and relative molar ratios of the *O*-linked oligosaccharides isolated from lamp-1 and lamp-2 and leukosialin from HL-60 cells.

Structure	Lamp-1	Lamp-2	Leukosialin[a]
GalNAcOH	3.4%	2.1%	13.3%
Galβ1→3GalNAcOH	5.1%	5.1%	2.9%
NeuNAcα2→3Galβ1→3GalNAcOH	22.7%	30.0%	7.1%
NeuNAcα2 ↘ 6 NeuNAcα2→3Galβ1→3GalNAcOH	-[b]	-	-
Galβ1→4GlcNAcβ1 ↘ 6 Galβ1→3GalNAcOH	3.0%	2.7%	4.8%
NeuNAcα2→3Galβ1→4GlcNAcβ1 ↘ 6 Galβ1→3GalNAcOH	4.5%		
Galβ1→4GlcNAcβ1 ↘ 6 NeuNAcα2→3Galβ1→3GalNAcOH	4.1%	14.3%	8.6%
NeuNAcα2→3Galβ1→4GlcNAcβ1 ↘ 6 NeuNAcα2→3Galβ1→3GalNAcOH	47.8%	37.3%	60.6%

HL-60, lamp-1, and lamp-2 contain approximately 21% and 12% of *O*-glycans as poly-*N*-acetyllactosaminyl *O*-glycans, respectively. [a]The data on leukosialin is in reference 17. [b]Less than 1%.

As shown previously, *O*-glycans are exclusively attached to the hinge-region of lamp-1 and lamp-2.[20] We therefore digested lamp molecules with trypsin (for lamp-1) or trypsin followed by V8 protease (for lamp-2). These digestion conditions allow us to obtain the whole hinge-region in one glycopeptide and yet excluding the *N*-glycosylation site (see Fig. 3 in reference 20). The glycopeptides obtained were then applied to a column of Jacalin-Agarose and the glycopeptides bound to this column were subjected to alkaline borohydride treatment. The amount of the glycopeptides bound to the Jacalin-column was about 50% more than the glycopeptides prepared after pronase. In order to remove *N*-glycans, the released glycans were applied to Con A-Sepharose and DSA-Agarose (Fig. 2B). *O*-Glycans unbound to these two lectins were then subjected to Bio-

Gel P-4 gel filtration. As shown in Fig. 3C and D, the oligosaccharides derived from the whole hinge-region are much more enriched in the disialosyl hexasaccharide (peak 3). The ratio of oligosaccharides 3-9 obtained from lamp molecules is almost the same as that obtained in leukosialin (Table I). These results indicate that the fully sialylated hexasaccharide was recovered in Jacalin-bound fraction because the glycopeptides contained small oligosaccharides that can be bound to Jacalin. The Jacalin-unbound fraction was desialyzed and again applied to the Jacalin column, but no additional glycopeptides were found, indicating that *O*-glycans were fully recovered in Fig. 3C and D.

Glycosidase Digestion of Poly-*N*-Acetyllactosaminyl *O*-Glycans - Peaks 1 and 2 obtained after Bio-Gel P-4 gel filtration (Fig. 3) were found to contain sialyl Le^x structures in poly-*N*-acetyllactosaminyl *O*-glycans, as summarized below.

(1) Peak 2 in Fig. 3A and B is larger than the disialosylhexasaccharide by one *N*-acetyllactosaminyl unit. This peak 2 eluted at the same position of the oligosaccharide NeuNAcα2→3Galβ1→4(±Fucα1→3)GlcNAcβ1→3Galβ1→4 GlcNAcβ1→6(NeuNAcα2→3Galβ1→3)GalNAcOH, which was isolated from leukosialin.[17]

(2) Both the peaks 1 and 2 were susceptible to endo-β-galactosidase (Fig. 4).

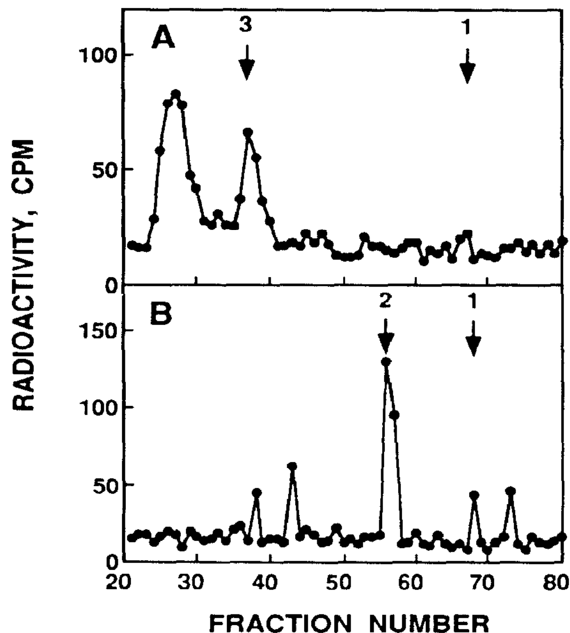

Fig. 4 Bio-Gel P-4 gel filtration of lamp-2 peak 1 after endo-β-galactosidase digestion.
Peak 1 in Fig. 3B was digested with endo-β-galactosidase before (A) and after desialylation (B). The elution positions of standard oligosaccharide are: 1, GlcNAcβ1→3Gal; 2, Galβ1→3(GlcNAcβ1→6)GalNAcOH plus Galβ1→4(Fucα1→3) GlcNAcβ1→3Gal; 4, NeuNAcα2→3Galβ1→4GlcNAcβ1→3Gal. Similar results were obtained on the lamp-1 sample.

(3) Both peak 1 and peak 2 are exclusively composed of sialylated oligosaccharides (Fig. 5A). Without prior treatment of sialidase, no release of Gal or GlcNAc was detected (data not shown). Peak 1 plus peak 2 yielded Galβ1→3GalNAcOH after digestion of neuraminidase, β-galactosidase and β-N-acetylglucosaminidase (Fig. 5B). This amount was increased when the oligosaccharides were digested with α1→3/4 fucosidase in addition to β-galactosidase and β-N-acetylglucosaminidase (Fig. 5C).

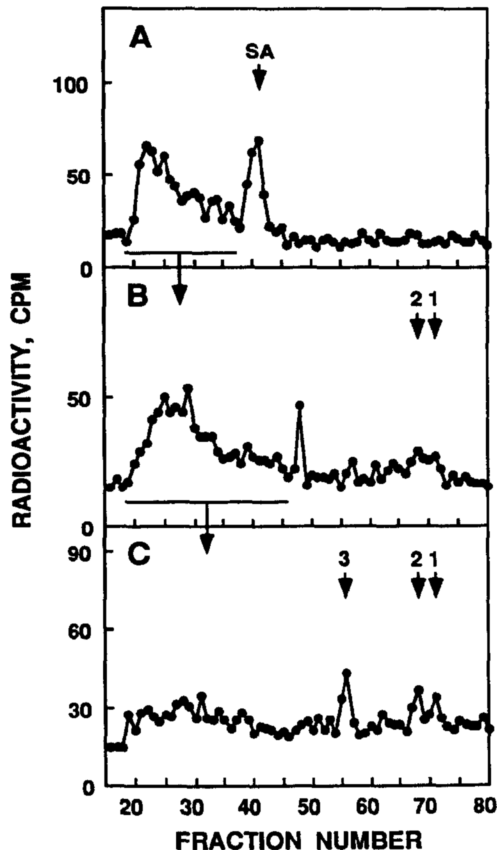

Fig. 5 Sequential glycosidase treatment of peak 1 plus peak 2 from lamp-1 and lamp-2.
Peak-1 plus peak 2 in Fig. 3C were digested with neuraminidase (A) then β-galactosidase plus β-*N*-acetylglucosaminidase (B) followed by β-galactosidase, β-*N*-acetylglucosaminidase plus α-fucosidase (C). The digest was subjected to Bio-Gel P-4 gel filtration. The elution position of the standard oligosaccharides are 1,GlcNAc; 2, Galβ1→3GalNAcOH; 3, $(Man)_3Fuc(GlcNAc)_2$. $(Man)_3Fuc(GlcNAc)_2$ was produced because alkalin borohydride treatment cleaved aspartylglycosylamine linkage.[35]

α1→3/4 Fucosidase cannot release fucose from sialylated form as follows

```
NeuNAcα2→3Galβ1→4GlcNAcβ1→R
                       3
                       ↑
                       α1
                       Fuc
        │
        ↓ α-Fucosidase
No cleavage
```

However, the same enzyme can release fucose from desialylated form.

```
NeuNAcα2→3Galβ1→4GlcNAcβ1→R
                       3
                       ↑
                       α1
        │              Fuc
        ↓ Neuraminidase
Galβ1→4GlcNAcβ1→R + NeuNAc
           3
           ↑
           α1
        │  Fuc
        ↓ α-Fucosidase
Galβ1→4GlcNAcβ1→R + Fuc
```

Since the *N*-acetylglucosamine residue can be released only after fucose is removed, the increased release of *N*-acetylglucosamine from the asialoform corresponds to sialyl Le^x structure. The results shown in Figure 5B and C thus demonstrated that approximately 65% of poly-*N*-acetyllactosaminyl *O*-glycans contain sialyl Le^x structure.

Figure 5C also shows that N-glycans originally present in peak 1 and peak 2 produced $(Man)_3Fuc(GlcNAc)_2$, peak 3 after this treatment. Since ^3H-glucosamine is incorporated into GalNAc and GlcNAc in a ratio of 0.7:1.0, the results indicate that *O*-glycans constitute slightly more than 60% of peak 1 and peak 2. Based on this calculation, poly-*N*-acetyllactosaminyl *O*-glycans were found to constitute 21% and 12% of the total radioactivity recovered in Bio-Gel P-4 gel filtration (Fig. 3C and D). These results indicate that 14% and 8% of *O*-glycans attached to lamp-1 and lamp-2 contain sialyl Le^x terminus.

Estimation of The Amount of Sialyl Le^x Structures Present in *N*-Glycans Attached to Lamp-1 and Lamp-2 - Previously, we have shown that some of *N*-glycans present in lamp-1 and lamp-2 contain sialyl Le^x structures at their termini.[19,29] However, the exact amount of the sialyl Le^x structure was not determined. Since we are now capable of determining the amount of sialyl Le^x structures, the attempt was made to determine the amount of sialyl Le^x structure in *N*-glycans. In order to simplify the analysis, poly-*N*-acetyllactosaminyl *N*-glycans were pooled into one fraction (IB, see Fig. 2A). On the other hand, *N*-glycans without *N*-acetyllactosaminyl repeats or with high content of fucose residues were recovered in the fraction unbound to both DSA-Sepharose and Jacalin-Agarose (IA1, Fig. 2A). In order to remove potentially contaminating *O*-glycans, the glycopeptides

were treated with alkaline borohydride. The results indicate only a very small amount of the radioactivity was due to *O*-glycans, shifting into low molecular weight after the treatment (Figs. 6A and 7A).

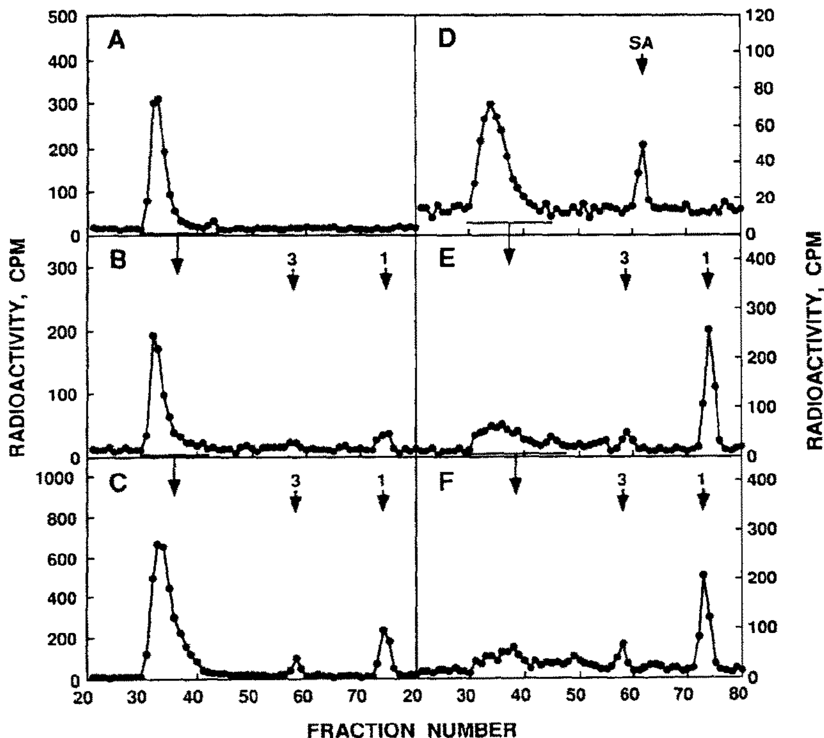

Fig. 6 Sequential glycosidase digestion of Jacalin unbound glycopeptides, IA1 obtained from lamp-1. Glycopeptides that were not bound to DSA-Agarose or Jacalin-Agarose (IA1 in Fig. 2A) were subjected to Bio-Gel P-4 gel filtration after alkaline borohydride treatment (A). The glycopeptides after alkaline borohydride treatment were then digested with β-galactosidase plus β-*N*-acetylglucosaminidase (B) followed by β-galactosidase, β-*N*-acetylglucosaminidase plus α-fucosidase (C). In parallel, the same glycopeptides after alkaline borohydride treatment were digested with neuraminidase (D), then by β-galactosidase plus β-*N*-acetylglucosaminidase (E) followed by β-galactosidase, β-*N*-acetylglucosaminidase plus α-fucosidase (F). The bars indicate the fractions pooled for further digestion. The elution positions of standard oligosaccharides are: 1, GlcNAc; SA, sialic acid; 3, $(Man)_3Fuc(GlcNAc)_2$. $(Man)_3Fuc(GlcNAc)_2$ was obtained because *N*-glycans were partially released from glycopeptides by alkaline borohydride treatment.[35]

The glycopeptides remaining after alkaline borohydride treatment were then subjected to sequential exoglycosidase digestion. Figure 6 illustrates that only a small portion of *N*-glycans in lamp-1 IA1 are susceptible to β-galactosidase plus β-*N*-acetylglucosaminidase prior to neuraminidase treatment. By comparing Fig. 6B and C, it can be estimated that 9.8% of *N*-acetylglucosamine is Galβ1→4GlcNAcβ1→3 and 11.2% is

Galβ1→4(Fucα1→3)GlcNAcβ1→3. Similar analysis was made after neuraminidase treatment (Fig. 6D and E), and it can be estimated that Galβ1→4GlcNAcβ1→3 constitutes 22.8% of *N*-acetylglucosamine whereas Galβ1→4(Fucα1→3)GlcNAcβ1→3 constitutes 24.8% in the desialylated sample. The difference thus obtained on the results before and after desialylation should yield the amount of sialylated forms. Thus lamp-1 IA-1 contains 13.0% of NeuNAcα2→3Galβ1→4GlcNAcβ1→3 and 13.6% of NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3.

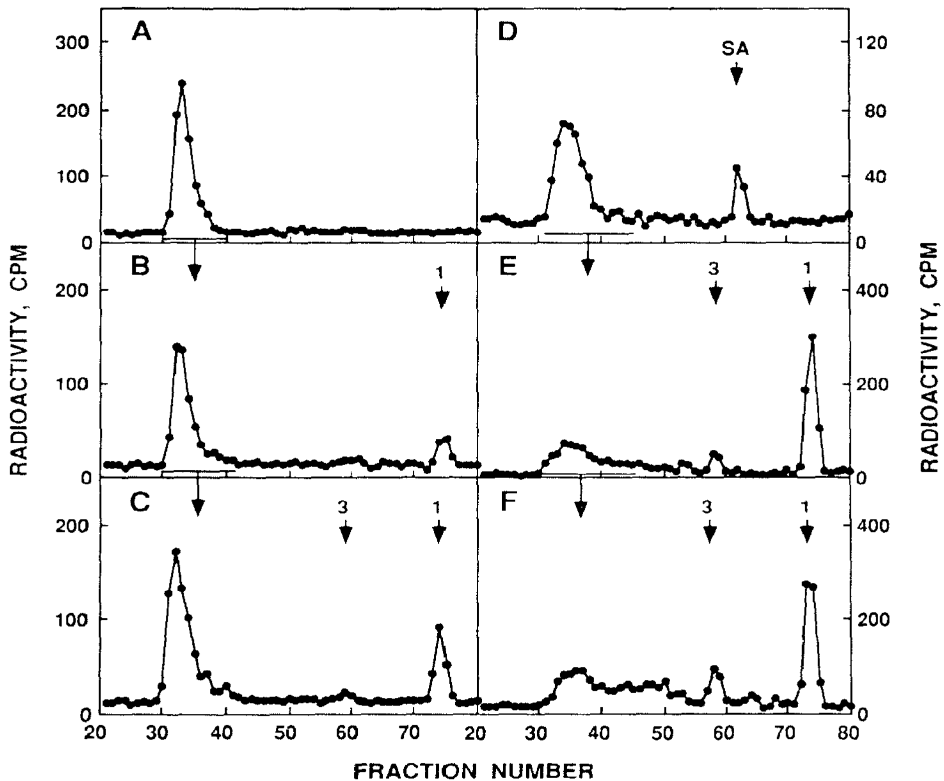

Fig. 7 Sequential glycosidase digestion of DSA-bound fraction (IB) from lamp-1.
Lamp-1 glycopeptides that were bound to DSA-Agarose (IB in Fig. 2A) were subjected to Bio-Gel P-4 gel filtration after alkaline borohydride treatment (A). The glycopeptides after alkaline borohydride treatment were then digested with β-galactosidase plus β-*N*-acetylglucosaminidase (B) followed by β-galactosidase, β-*N*-acetylglucosaminidase plus α-fucosidase (C). In parallel, the glycopeptides after alkaline borohydride treatment were digested with neuraminidase (D), then β-galactosidase plus β-*N*-acetylglucosaminidase (E) followed by β-galactosidase, β-*N*-acetylglucosaminidase plus α-fucosidase (F). The elution positions of standard oligosaccharides are as shown in Fig. 6. The bar indicates the fractions pooled for further digestion.

We have previously shown[19] that IA1 fraction contains a total of 6.5 moles of *N*-acetylglucosamine. It was also determined that the IA1 fraction contains 4.2 moles of *N*-glycans/mole of lamp-1, judging from the recovery of glycopeptides in each fraction and the total number of *N*-glycans.[19] Based on this data, it can be calculated that the IA1 fraction in the present study represents the total of 27.3 moles of *N*-acetylglucosamine residues (6.5 x 4.2 = 27.3). Based on this total amount of *N*-acetylglucosamine and the amount of each structure determined above, the amount of Galβ1→4GlcNAcβ1→3, Galβ1→4(Fucα1→3) GlcNAcβ1→3, NeuNAcα2→3Galβ1→4GlcNAcβ1→3, and NeuNAcα2→3Galβ1→4 (Fucα1→3)GlcNAcβ1→3, can be calculated.

Similar calculations can be made on DSA-bound fraction (IB) obtained from lamp-1, based on the yield of *N*-acetylglucosamine after sequential glycosidase digestion (Fig. 7). The amount of each structure in lamp-1 was obtained by summation of those present in IA1 and IB and summarized in Table II. Based on similar experiments, the amount of each structure can be calculated on lamp-2 (Table II).

TABLE II
The amount of *N*-acetyllactosamines with Le^x or sialyl Le^x terminus in lamp-1 and lamp-2 of HL-60 cells.

Structure	Lamp-1		Lamp-2		Leukosialin[a]
	N-glycans	*O*-glycans	*N*-glycans	*O*-glycans	*O*-glycans
			moles/mole of glycoproteins		
(Galβ1→4GlcNAcβ1→3)$_n$	12.3[b]	0	11.0	0	0
(Galβ1→4GlcNAcβ1→3)$_n$ 3 ↑ α1 Fuc	13.4	0	11.8	0	0
NeuNAcα2→3(Galβ1→4GlcNAcβ1→3)$_n$	26.6	0.6	31.4	0.3	0.7
NeuNAcα2→3(Galβ1→4GlcNAcβ1→3)$_n$ 3 ↑ α1 Fuc	4.7	1.1	3.2	0.7	0.5

a. The data on leukosialin is in reference 17. b.The values are based on the numbers of *N*-glycans and *O*-glycans attached to each molecule and obtained by calculation of the yield of each structure.

DISCUSSION

The present studies demonstrated that *O*-glycans present in lysosomal membrane glycoproteins, lamp-1 and lamp-2, contain poly-*N*-acetyllactosaminyl repeats when the proteins were isolated from HL-60 cells. The studies also demonstrated that most of these poly-N-acetyllactosaminyl *O*-glycans are terminated with the sialyl Le^x structure, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc→R. The results also show that the amount of sialyl Le^x in *O*-glycans is significant, considering that only a fraction of *N*-glycans contain the sialyl Le^x terminus (Table II).

Previously, we have shown only 1.5% of *O*-glycans attached to leukosialin contain poly-*N*-acetyllactosaminyl extension and 40% of those poly-*N*-acetyllactosaminyl *O*-glycans contain sialyl Le^x terminus. Since leukosialin contains approximately 80 *O*-glycans per molecule, these results indicate that leukosialin contains, on average, 0.5 mole of sialyl Le^x terminus. In contrast, despite the fact that only 5 to 8 *O*-glycans are present in lamp molecules, *O*-glycans in lamp molecules contain slightly more sialyl Le^x terminus per molecule than leukosialin does. This is mostly because *O*-glycans attached to lamp molecules contain about fifteen times more poly-*N*-acetyllactosaminyl repeats than leukosialin. Lamp molecules were isolated in our laboratory as the major sialoglycoproteins containing *N*-acetyllactosamine repeats in *N*-glycans.[18] The present study thus demonstrated that both *N*- and *O*-glycans in lamp molecules are modified by poly-*N*-acetyllactosamine extension more efficiently than those attached to leukosialin. These results support the previous findings that poly-*N*-acetyllactosamine formation is somehow intrinsic to the nature of glycoproteins.[1,2] Poly-*N*-acetyllactosamines are formed when β-1,3-*N*-acetylglucosaminyltransferase and β-1,4-galactosyltransferase alternatively add *N*-acetylglucosamine and galactose to *N*-acetyllactosamine, the precursor (Fig. 8). We found that glycoproteins containing a significant amount of poly-*N*-acetyllactosamine go through the Golgi complex slowly so that the enzymes adding *N*-acetyllactosamine repeats can act on those glycoproteins.[30] It is also possible that lamp molecules present the precursor carbohydrate in a unique way so that *N*-acetyllactosamine repeats are efficiently formed by alternate additions of *N*-acetylglucosamine and galactose.

It has been shown previously that leukosialin from HL-60 cells contains sialyl Le^x terminus but not leukosialin from K562 cells.[17] *O*-Glycans from HL-60 cells contain those based on core 2 branchings which are formed by core 2 β-1,6-*N*-acetylglucosaminyltransferase and those branched oligosaccharides can be modified to acquire poly-*N*-acetyllactosaminyl repeats when β-1,3-*N*-acetylglucosaminyltransferase is also present (Figure 8). Moreover, those poly-*N*-acetyllactosaminyl extensions apparently acquire sialyl Le^x terminus much more efficiently than one *N*-acetyllactosaminyl branch attached to Galβ1→3GalNAc. This is probably because α-1,3-fucosyltransferase adds more efficiently a fucose to sialyl *N*-acetyllactosamine when the terminal structure is present at the end of poly-*N*-acetyllactosamine extension.

In order to form sialyl Le^x structure in *O*-glycans, the formation of core 2 branch is thus a prerequisite. In fact, we have shown recently that the expression of cloned core 2 β-1,6-*N*-acetylglucosaminyltransferase made Chinese hamster ovary cells express poly-*N*-acetyllactosamine extensions in *O*-glycans.[31] The parent CHO cells lacking this enzyme were found not to contain poly-*N*-acetyllactosaminyl *O*-glycans. These results clearly indicate that core 2 β-1,6-*N*-acetylglucosaminyltransferase is the key enzyme for sialyl Le^x terminus in *O*-glycans (see Figure 8).

In relation to this, it is noteworthy that human T-cell activation was found to be associated with the appearance of core 2-based oligosaccharides.[16] It was found later that sialyl Le^x structure appears on T cells

when they are activated.[32] These results strongly suggest that core 2 formation is also the key step for sialyl Le^X formation in activated T-lymphocytes.

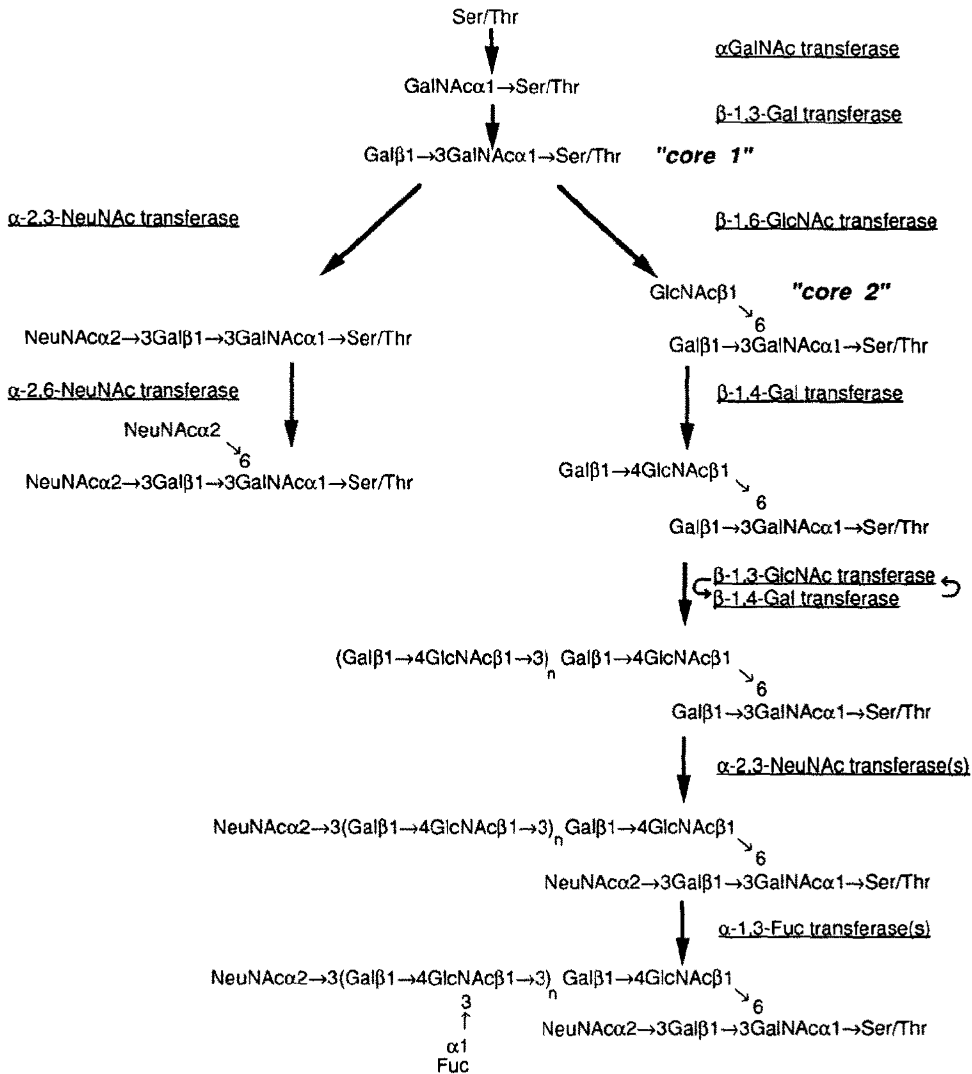

Fig. 8 The proposed biosynthetic pathways of *O*-glycans and poly-*N*-acetyllactosaminyl *O*-glycans.
It has been shown that the tetrasaccharide (bottom left) is formed by sequential addition of α2→3 sialyltransferase followed by α2→6 sialyltransferase. When β1→6 *N*-acetylglucosaminyltransferase, core 2 enzyme, is present, the core 2 branch is formed. To this core 2 branch, poly-*N*-acetyllactosaminyl extension can be added. Poly-*N*-acetyllactosaminyl chain can be further modified by α1→3 fucosyltransferase, forming sialyl Le^X terminus (based on Refs. 6, 12, 17, 26 and 31).

It has been demonstrated in several laboratories that the sialyl Le^x structure is the ligand for E- and P-selectins.[6-9] Recent studies revealed that sialyl Le^x structure is more efficiently recognized by selectin when they are present in mucin-type glycoproteins.[33,34] The results strongly suggest that presentation of ligands in *O*-glycans leads into higher affinity to selectins because they are present as a cluster. Since the formation of sialyl Le^x in *O*-glycans is determined by core 2 branchings, further studies are significant to understand how the expression of core 2 forming β-1,6-*N*-acetylglucosaminyltransferase is regulated.

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