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Alpha 1,6-Linked Fucose Affects the Expression and Stability of Polysialic Acid-Carrying Glycoproteins in Chinese Hamster Ovary Cells[1]

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To determine the effect of α1,6-linked fucose modification of *N*-glycans on the expression of polysialic acids (PSAs), the expression of PSAs in a fucose-lacking mutant of Chinese hamster ovary (CHO) cells, Lec13, was compared with that in CHO K1 cells. PSA synthase activity in these cells and the antennary structures of *N*-glycans associated with the neural adhesion molecule (NCAM), which is a major PSA-carrying glycoprotein, did not differ between the two types of cells. Metabolic labeling of cells with [^{3}H]glucosamine for 48 h followed by immunoprecipitation with anti-PSA monoclonal antibodies revealed that the amount of labeled PSA-carrying glycoproteins obtained from Lec13 cells was 10-times less than that from K1 cells, although the incorporation of [^{3}H]glucosamine into total extracts and NCAM was almost the same. In contrast, when cells were pulse labeled with [^{35}S]-methionine followed by a 1 h chase, there was not such a great difference in PSA-carrying protein synthesis between K1 and Lec13 cells. However, during a prolonged chase period, PSA-carrying proteins rapidly decreased in Lec13 cells, whereas those in K1 cells did not change. The degradation of PSA-carrying glycoproteins in Lec13 cells was partly prevented when the cells were grown in fucose-containing medium. Therefore, fucose modification of core *N*-glycans may affect the efficient expression of PSAs through the intracellular stability of PSA-carrying glycoproteins.

Key words: alpha 1,6-linked fucose, Chinese hamster overy cells, neural cell adhesion molecule, *N*-glycans, polysialic acid.

A polysialic acid (PSA) is a unique homopolymer of α2,8-linked sialic acids and is mainly associated with the neural cell adhesion molecule (NCAM) in mammals (*1, 2*). Expression of PSAs on NCAM has been shown to be highly regulated, *i.e.*, NCAM is much more abundantly polysialylated in fetal brain than adult brain (*1*). The biological significance of PSAs on NCAM in pathfinding and targeting as to the innervation of axons, migration of neuronal cells and tumor cells, and spatial learning and memory has been described (*2*). However, only little is known about how polysialylation is regulated during brain development or why NCAM is selectively polysialylated. Recently, cDNAs encoding two distinct PSA synthases, ST8Sia II and IV, were cloned and characterized (*3-10*), and one of them, ST8Sia II, was shown to be highly regulated during brain development and neuronal differentiation (*7, 11-13*), suggesting that regulation of PSA expression is partly directed by PSA synthase gene expression. On the other hand, it has been shown that the alterations in the extracellular polypeptide structure of NCAM caused by alternative splicing affected the polysialylation of NCAM (*14-17*), suggesting that a particular protein conformation is required for the expression of PSAs on certain glycoproteins. More recently, the structure of the *N*-glycan core which carries PSAs was re-characterized and shown to contain several unique elements, such as α1,6-linked fucose

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Abbreviations: The abbreviated nomenclature for the cloned sialyltransferases follows the system of Tsuji *et al.* (*45*). ST8Sia II, the same polysialic acid synthase as STX; ST8Sia IV, the same polysialic acid synthase as PST-1 cloned from hamster (*3*); CHO, Chinese hamster ovary; PSA, polysialic acid; NCAM, neural cell adhesion molecule; NCAM-Fc, soluble recombinant NCAM fused with the Fc region of human IgG; endo N, endoneuraminidase; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; EDTA/PBS solution, PBS containing 0.53 mM EDTA; BSA, bovine serum albumin; mAb, monoclonal antibody; pAb, polyclonal antibody; PMSF, phenyl methylsulfonylfluoride; MES, 2-[*N*-morpholino]ethanesulfonic acid; Con A, concanavalin A; LCA, lentil lectin; DSA, *Datura stramonium* agglutinin; $GlcNH_2$, glucosamine; Met, methionine; Sia, sialic acid; PNGase, peptide:*N*-glycanase.

attached to the proximal GlcNAc of the di-*N*-acetyl chitobiose unit and sulfate residues (*18, 19*). In addition, *in vitro* experiments involving soluble recombinant ST8Sia II and IV demonstrated that the α1,6-linked fucose attached to the di-*N*-chitobiose unit of *N*-glycan partly contributed to the PSA formation (*9*). Thus, it is also possible that unique elements, such as α1,6-linked fucose, may play roles in the determination of PSA expression on certain *N*-glycans.

CHO cells are known to express the polysialylated form of NCAM, and a PSA synthase, ST8Sia II, was cloned from a CHO cell line (*3*). In addition, it was shown that ST8Sia IV is the only factor for PSA synthesis and expression in CHO cells (*20*). Lec13 cells were established as a pea-lectin resistant CHO mutant (*21*) and shown to lack GDP-mannose 4,6-dehydrogenase activity, therefore the cells can not synthesize GDP-fucose, which is the precursor for fucose residues in complex carbohydrates (*22*). It was also shown that the complex carbohydrates produced by Lec13 cells lack α1,6-linked fucose (*22*). Thus, this α1,6-linked fucose-lacking CHO mutant, Lec13, provides us with an *in vivo* model for studying the role of the α1,6-linked fucose modification of complex carbohydrates during PSA synthesis and expression. Recently, it was reported that α1,6-fucose is not essential for *in vitro* PSA synthesis because PSAs were synthesized on α1,6-linked fucose lacking NCAM-Fc (soluble recombinant NCAM fused with the Fc region of human IgG) as well as α1,6-linked fucose containing NCAM-Fc by recombinant PSA-synthases (*23*). In this study, we focus on the *in vivo* biosynthesis and expression of PSAs using Lec13 cells. We compare the *in vivo* synthesis of PSAs between Lec13 cells and the parental CHO K1 cells, and demonstrate that PSAs are expressed much more efficiently in CHO K1 cells than in fucose-lacking Lec13 cells.

MATERIALS AND METHODS

Cells and Antibodies—The Chinese hamster ovary (CHO) cell line, K1, was obtained from the RIKEN GENE BANK. The pea-lectin resistant CHO mutant cell line, Lec13, which lacks the synthetic pathway from GDP-Man to GDP-fucose (*21, 22*), was kindly provided by Dr. Pamela Stanley (Albert Einstein College of Medicine). Both types of cells were cultured in minimum essential alpha medium (GIBCO) supplemented with 10% fetal bovine serum. The anti-polysialic acid (PSA) monoclonal antibody (mAb) (mouse IgG_1), 735 (*24*), was a gift from Dr. Rita Gerardy-Schahn (Inst. für Med. Mikrobiologie, Hanover, Germany). Antibody 735 was shown to react with polysialic acid chains containing eight or more α2,8-linked sialic acid residues (*25*). The anti-neural cell adhesion molecule (NCAM) mAb (mouse IgG_1), produced using E17 rat forebrain as an immunogen, NCAM-OB11, was purchased from Sigma. The anti-NCAM polyclonal rabbit antibody, NA-1206, was purchased from Affinity Research Products. We confirmed that these antibodies could be used for quantitative immunoprecipitation of NCAM prepared from mouse and hamster. However, only 30-50% of the polysialylated form of NCAM could be immunoprecipitated with NCAM-OB11 or NA-1206. In addition, NCAM antibodies have been shown to react with NCAM with high molecular PSAs much more weakly than with nonpolysialylated NCAM (see Fig. 1) (*26*). Endoneuraminidase (*27*) was kindly provided by Dr. Frederick A. Troy (University of California). Anti-Le^x mAb (clone 73-30, IgM), anti-Le^y mAb (clone H18A, IgG3), and FITC-conjugated *Ulex europaeus* agglutinin were purchased from Seikagaku. Unless otherwise indicated, the materials and methods are essentially the same as described previously (*9-11*).

Western Blotting—For immunoblot analysis, cells (3×10^6) were sonicated on ice in a lysis buffer (50 mM MES, pH 6.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF), incubated for 15 min at 4°C with gentle rotation, and centrifuged at $15,000\times g$ for 15 min, and then the protein concentration of the supernatant was measured by the BCA method (Pierce). The lysate (20 μg of protein) was subjected to SDS-PAGE on a 5-20% gel. The proteins were then transferred to a Zetaprobe (Bio-Rad) filter paper. The filter paper was blocked for 60 min with PBS containing nonfat dry milk, and then incubated with an anti-NCAM antibody, NA-1206 (1:1,000 dilution), and then anti-PSA mAb 735, overnight at 4°C, followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG goat and then anti-mouse IgG rabbit secondary antibodies. NCAM and PSAs were detected as to chemiluminescence using a Phototope™-Star detection kit (New England BioLabs).

Labeling of Cells—CHO K1 and Lec13 cells (0.6×10^6) were seeded into 6 cm-diameter culture dishes containing 2 ml alpha medium supplemented with 10% FCS, and then 100 μCi of [^{3}H]$GlcNH_2$ (50 μCi/ml) was added to the medium. After 48 h, the cells had proliferated to $2.3\text{-}2.8\times10^6$ (in the case of K1 cells) and $2.6\text{-}3.0\times10^6$ (in the case of Lec13 cells). The labeled cells were harvested, washed with PBS, and then incubated with 200 μl of another lysis buffer (50 mM MES, pH 6.0, containing 0.15 M NaCl, 1.0% Nonidet P-40, 5 mM EDTA, and 1 mM PMSF) at 4°C for 30 min with gentle rotation. The cells were then centrifuged and the supernatant (cell lysate) was collected. For the immunoprecipitation of PSA-carrying glycoproteins, the lysate of 1×10^6 cells was incubated with 10 μg/ml of anti-PSA mAb, 735, for 2 h. Protein G-Sepharose (10 μl of resin) was then added to the mixture and the mixture was gently rotated at 4°C for overnight. The immune complex adsorbed to protein G-Sepharose was recovered by centrifugation, and the resin was washed 4 times with the extraction buffer and twice with PBS. For the immunoprecipitation of NCAM, the cell lysate was first treated with neuraminidase F (0.5 U/ml) for 3 h, and then the treated cell lysate was incubated with anti-NCAM mAb, NCAM-OB11 (1:100 dilution), for 2 h. The radioactivity in an aliquot of the resin was counted, the rest of it being analyzed by SDS-PAGE on a 5-20% gradient gel before and after treatment with neuraminidase F (0.5 U/ml) for 3 h, followed by autoradiography.

For pulse-chase labeling with [^{35}S]Met, cells (0.4×10^6) were cultivated in the wells of a 6-well culture plate (Falcon), washed twice with methionine-free DMEM, and then incubated with the same medium (0.5 ml) for 30 min. The cells were then pulse-labeled with [^{35}S]Met (100 μCi/ml) in methionine-free DMEM for 10 min at 37°C. After the pulse-labeling, non-radiolabeled methionine (1 mM) was added to the medium, the medium was changed to alpha medium supplemented with 10% FCS and 1 mM methionine, and then the cells were cultured for 1, 2, 4, or 8 h. After the cells had been washed with PBS twice, they

were lysed with the lysis buffer. The cell lysate was centrifuged with 15,000 rpm for 20 min. The supernatant was precleared with nonimmunized mouse IgG and protein G-Sepharose for 16 h at 4°C with gentle rotation, and then the resulting supernatant was immunoprecipitated with anti-NCAM mAb, NCAM-OB11, or anti-PSA mAb, 735, and protein G-Sepharose, as described above. The beads were collected by centrifugation and then subjected to SDS-PAGE on a 5% acrylamide gel before and after treatment with neuraminidase F. The gel was fixed and dried, and radioactivity was detected with a BAS2000 radio image analyzer (Fuji Film).

Flow Cytometry—For flow cytometry, cells were freshly harvested using an EDTA/PBS solution (PBS containing 0.53 mM EDTA), and then incubated with anti-PSA mAb for 60 min at 4°C, followed by FITC-conjugated anti-mouse IgG+M for 45 min at 4°C. The fluorescence intensity of the cells was analyzed using an FACSort (Becton Dickinson). To determine the time course of PSA expression, the cells (5×10^6) were harvested with the EDTA/PBS solution, suspended in 100 μl of PBS, and then treated with endoneuraminidase (1:10 dilution), which cleaves poly-α2,8-sialic acids, for 1 h at 37°C. After this treatment, the cells were washed with PBS, and then one-fifth of the cells (1×10^6) was fixed with 4% paraformaldehyde for 1 h at 4°C, washed with PBS, and stored at 4°C until analysis. 1×10^6 of the remaining cells were seeded into a 6-cm-diameter culture dish and cultured for 15, 40, 60, or 72 h. After the incubation, the cells were harvested using the EDTA/PBS solution and then fixed with 4% paraformaldehyde. The fixed cells were treated with 1% BSA-containing PBS for 1 h at 4°C and then stained with anti-PSA mAb or nonimmunized mouse IgG, followed by analysis of PSA expression with the FACSort.

Assay for PSA Synthase Activity—PSA synthase activity in cells was measured using soluble recombinant NCAM fused with the Fc region of human IgG (NCAM-Fc) as the acceptor substrate and the membrane fraction of the cells as the enzyme source, as described previously (*13*). Briefly, the reaction mixture, comprising 0.1 mM CMP-[^{14}C]Sia (0.25 μCi), 10 mM $MgCl_2$, 25 mM MES, pH 6.0, 0.5 μg of NCAM-Fc, and 50 μg of membrane protein, was incubated at 37°C for 4 h. Then NCAM-Fc in the reaction mixture was recovered by adding protein A-Sepharose, followed by SDS-PAGE. PSA synthase activity was estimated as the difference in radioactivity between before and after treatment with endoneuraminidase. As a control, the incorporation of sialic acids into asialo α1-acid glycoprotein (20 μg, 1 mg/ml) was also measured under the same conditions as for the α2,3 and α2,6-sialyltransferase activities.

Preparation and Analyses of Glycopeptides Derived from NCAM and PSA-Carrying Glycoproteins—Cells were labeled with [^{3}H]$GlcNH_2$, and then the labeled glycoproteins were extracted from the cells, as described above. The labeled NCAM and PSA-carrying glycoproteins were immunoprecipitated with anti-NCAM mAb and anti-PSA mAb, respectively. The glycopeptides of NCAM or PSA-carrying glycoproteins were prepared by digestion with pronase K (100 μg/ml) in 50 mM Tris-HCl, pH 8.0, at 65°C for 36 h. Pronase K was used after preincubation at 65°C for 1 h for inactivation of glycosidase activity. After desalting by passage through a G-25 column equilibrated with water, the glycopeptides derived from NCAM or PSA-carrying glycoproteins were separated on a DEAE-Toyopearl column (Toso) with a linear gradient of ammonium acetate (0–0.6 M). Fractions (1 ml) were collected and aliquots were counted with a liquid scintillation counter. The materials eluted at around 0.1 M ammonium acetate and 0.4 M ammonium acetate were collected and designated as non-PSA-carrying glycopeptides and PSA-carrying glycopeptides, respectively.

For lectin affinity chromatography of glycopeptides prepared from NCAM, a labeled cell lysate was first treated with neuraminidase F, and then NCAM was immunoprecipitated with anti NCAM mAb, followed by digestion with pronase K. In this case, all glycopeptides were eluted at under 50 mM ammonium acetate. Lectin affinity column chromatography was carried out according to the method described by Kobata and Yamashita (*28*). Briefly, the glycopeptides were dissolved in 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% NaN_3 (TB), and then applied to a concanavalin A (Con A)-agarose column (bed volume, 2 ml) equilibrated with TB. The column was washed with TB, and then eluted with 5 column volumes each of 5 mM methyl-α-mannoside in TB and 0.2 M methyl-α-mannoside in TB. Fractions (2 ml) were collected and aliquots of 100 μl were counted with a liquid scintillation counter. The materials that passed through the column (Con A-agarose unbound materials), and those eluted at 5 mM methyl-α-mannoside (Con A-agarose bound materials) were pooled and desalted, respectively. Half of the Con A-agarose unbound materials was then applied to a *Datura stramonium* agglutinin (DSA)-agarose column (bed volume, 2 ml) equilibrated with TB. The column was washed with 15 bed volumes of TB and then eluted with 1% *N*-acetylchitotetraose in TB. Fractions (2 ml) were collected and aliquots of 100 μl were counted with a liquid scintillation counter. The rest of the Con A-agarose unbound materials or Con A-agarose bound materials was applied to a lentil lectin (LCA)-agarose column (bed volume, 2 ml) equilibrated with TB. The column was washed with 10 volumes of TB and then eluted with 0.2 M methyl-α-mannoside in TB. Fractions (2 ml) were collected and aliquots of 100 μl were counted with a liquid scintillation counter. All the lectin-conjugated agarose was purchased from Honen, and all procedures were carried at 4°C.

Cultivation with L-Fucose—The cells were maintained in 1, 2, or 5 mM L-fucose containing alpha medium for at least 72 h before use. The medium was changed every 2 days. For pulse-chase labeling of cells cultured with fucose, 2 mM L-fucose-containing medium was used. For determination of the expression of Fucα1,2Gal and Fucα1,3GlcNAc structures in Lec13 cells after cultivation with fucose, cells were incubated with anti-Le^x mAb or anti-Le^y mAb for 60 min at 4°C, followed by FITC-conjugated anti-mouse IgG+M for 45 min at 4°C, or incubated with FITC-conjugated *Ulex europaeus* agglutinin for 30 min at room temperature, and then expression of the carbohydrate structures was determined by fluorescence microscopy.

Transfection of ST8Sia II cDNAs into Cells—CHO cells are known to express ST8Sia IV but not ST8Sia II mRNA. To establish highly PSA-expressing CHO cells, cDNA containing the full open reading frame of mouse ST8Sia II was ligated into the cloning site of the mammalian expression vector, pCAGSS, including hygromycin resistance,

yielding pCAGSS-mST8Sia II. The plasmid was transfected into CHO K1 and Lec13 cells by means of lipofectoamine (*9, 10*). After culturing for 72 h in DMEM supplemented with 10% FCS, cells were selected in 0.7 mg/ml hygromycin. mRNA was prepared from hygromycin-resistant colonies and expression of ST8Sia II mRNA was determined. Fourteen and five mST8Sia II mRNA-expressing clones were obtained from K1 and Lec13 cells, respectively. PSA expression of the clones was determined by flow cytometry and Western blotting using anti-PSA mAb, and the highest PSA-expressing clones derived from K1 and Lec13 cells, which were designated as K1-II and Lec-II, respectively, were examined in this study.

RESULTS

Comparison of De Novo Synthesis of PSAs between Lec13 and K1 Cells—The fucose-lacking CHO mutant, Lec13 cells, like CHO K1 cells, expresses PSAs (Fig. 1A), but the expression level was lower than that in CHO K1 cells based on the immunofluorescence intensity after cells had been stained with anti-PSA mAb (Fig. 1B). The highly sialylated NCAM is much larger than that detected with anti-NCAM antibodies (Fig. 1A), consistent with the results reported previously (*26*), because anti-NCAM antibodies have been shown to react with NCAM with high molecular PSAs much more weakly than with nonpolysialylated NCAM. The PSA synthase activities in Lec13 and K1 cells were not significantly different, *i.e.*, 74.2 and 94.5 pmol/mg protein·h, respectively. In addition, the α2,3- and α2,6-sialyltransferase activities in Lec13 cells were almost the same as those in K1 cells, *i.e.*, 150 and 145 pmol/mg protein·h in Lec13 and K1 cells, respectively. It is possible that this small difference in PSA synthase activity between the two types of cells may cause the difference in PSA expression. To rule out this possibility, another PSA-synthase cDNA (mST8Sia II) was introduced into the cells and the PSA-synthase was overexpressed. Both cell lines expressed ST8Sia IV mRNA, but not ST8Sia II mRNA, as a PSA synthase mRNA (data not shown). The PSA-synthase activities in mST8Sia II cDNA-transfected Lec13 and K1 cells (designated as Lec-II and K1-II cells, respectively, in this paper) were 651.8 and 810.1 pmol/mg protein·h, respectively. PSA expression in both types of cells increased as compared with in the respective parental cells.

To investigate the roles of fucose modification of the *N*-glycans in PSA synthesis and expression *in vivo*, the *de novo* synthesis of PSA in the two cell lines was compared by metabolic labeling with [^{3}H]GlcNH$_2$, followed by immunoprecipitation with anti-PSA mAb, 735. During the labeling period (48 h), the total radioactivities incorporated into Lec13 and K1 cells were almost the same (Table I). The migration patterns on SDS-PAGE of the labeled glycoproteins in Lec13 and K1 cells were very similar (Fig. 2). The *de novo* incorporation of [^{3}H]GlcNH$_2$ into NCAM was compared between before and after cell lysates had been treated with sialidase, followed by immunoprecipitation with anti-NCAM mAb, since NCAM is known to be a major carrier of PSAs in CHO cells. As shown in Fig. 2 and Table I, the radioactivity incorporated into NCAM of Lec13 and K1 cells was almost the same. In contrast, the radioactivity recovered in the immunoprecipitate obtained with anti-PSA mAb from Lec13 cells was 10-times less than that from K1 cells (Fig. 2 and Table I), although both types of cells synthesized PSAs. After being treated with sialidase, the majority (over 90%) of PSA-carrying glycoproteins was re-immunoprecipitated with anti-NCAM mAb and gave a

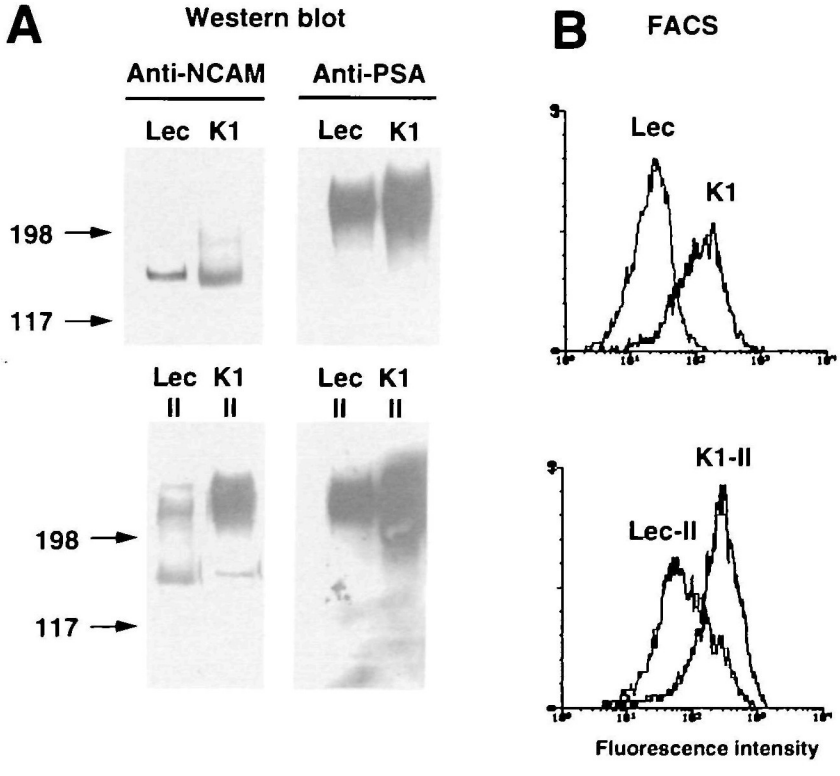


Fig. 1. **Expression of polysialic acids in CHO cells.** A: Cells were sonicated on ice in 50 mM MES, pH 6.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF, and then incubated for 15 min at 4°C with gentle rotation. The lysate (20 μg of protein) was subjected to SDS-PAGE on a 5-20% gel. The proteins were then transferred to a Zetaprobe (Bio-Rad) filter paper. The filter paper was blocked for 60 min with PBS containing nonfat dry milk, and then incubated with anti-NCAM pAb, NA-1206 (1:1,000 dilution), overnight at 4°C, followed by incubation with the alkaline phosphatase-conjugated anti-mouse IgG rabbit secondary antibody. B: Cells were freshly harvested using an EDTA/PBS solution, and then incubated with anti-PSA mAb for 60 min at 4°C, followed by FITC-labeled anti-mouse IgG+M for 45 min at 4°C. The fluorescence intensity of the cells was determined with a FACSort (Becton Dickinson).

140 kDa protein on SDS-PAGE (data not shown). Therefore, most of the PSA-carrying glycoproteins in CHO cells were most probably NCAM-140. If mST8Sia II cDNA-transfected Lec13 cells, in which the PSA synthase activity was 7-fold higher than that in parental K1 cells, were used for the metabolic labeling with [^{3}H]$GlcNH_2$, the radioactivity recovered in PSA-carrying glycoproteins was lower than that in the case of K1 cells without transfection of mST8Sia II cDNA (Table II and Fig. 2B). Thus, the PSA synthesis and expression in Lec13 cells were quantitatively different from those in K1 cells.

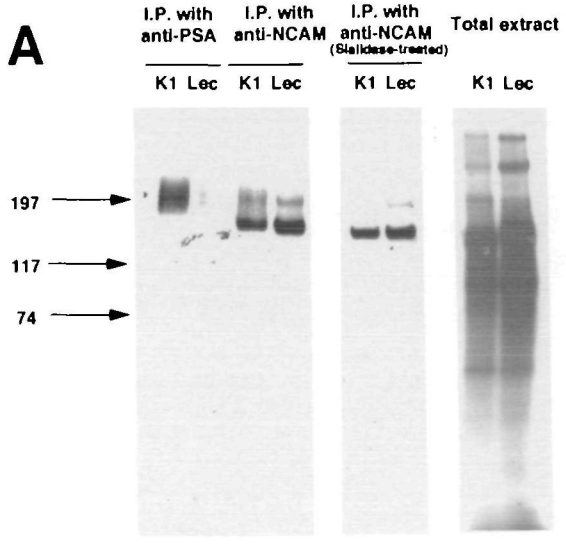
Polysialylation in Lec13 Cells Is Qualitatively Different from That in K1 Cells—We then investigated whether or

TABLE I. **Incorporation of [^{3}H]$GlcNH_2$ into PSA-carrying glycoproteins and NCAM in K1 and Lec13 cells.** Cells were labeled with [^{3}H]$GlcNH_2$ for 48 h, and then lysed with a lysis buffer. PSA-carrying glycoproteins were isolated by immunoprecipitation with anti-PSA mAb. NCAM was isolated by immunoprecipitation with anti-NCAM mAb after the cell lysate had been treated with sialidase. Aliquots of the immunoprecipitates absorbed to protein G-Sepharose resin were counted after the resin had been washed 4 times with the lysis buffer and 2 times with PBS. K1-II and Lec-II indicate mST8Sia II cDNA-transfected K1 and Lec13 cells, respectively. K1+Fuc and Lec13+Fuc indicate cells grown in 2 mM L-fucose containing medium. Each value represent the mean±SD for three independent experiments. The values in parentheses indicate the ratios of the radioactivity incorporated into the cells to that into CHO K1 cells.

	Radioactivity in 1×10^6 cells (cpm)		Total extract
	Anti-PSA I.P.	Anti-NCAM I.P.	
K1	14,400±2,800 (1.00)	17,500±4,400 (1.00)	2.2×10^8 (1.00)
Lec13	1,790±580 (0.12)	20,840±1,900 (1.19)	2.5×10^8 (1.13)
K1-II	48,100±6,000 (3.34)	19,560±3,800 (1.12)	2.5×10^8 (1.13)
Lec-II	5,530±2,200 (0.38)	14,500±2,600 (0.82)	2.4×10^8 (1.09)
K1+Fuc	12,600±1,380 (0.88)	15,220±890 (0.87)	2.0×10^8 (0.91)
Lec13+Fuc	4,180±2,800 (0.29)	12,850±1,730 (0.73)	1.9×10^8 (0.86)

TABLE II. **Populations of polysialylated glycopeptides in PSA-carrying glycoprotein preparations derived from K1 and Lec13 cells.** Cells were labeled with [^{3}H]$GlcNH_2$ for 48 h, and then lysed. PSA-carrying glycoproteins were prepared by immunoprecipitation with anti-PSA mAb. Three individual immunoprecipitates were pooled and used for the experiment in each case. The total radioactivity in PSA-carrying glycoproteins prepared from K1 and Lec13 cells, and mST8Sia II cDNA-transfected ones (K1-II and Lec-II) was about 51,000, 5,200, 58,000, and 13,000 cpm, respectively. PSA-carrying glycoproteins were also prepared from mST8Sia II cDNA-transfected Lec13 and K1 cells. The glycopeptides were prepared by digestion with pronase K and then separated on a DEAE-Toyopearl column, as described under "MATERIALS AND METHODS." The materials eluted with about 0.1 M ammonium acetate and 0.4 M ammonium acetate, as shown in Fig. 3, were collected and designated as non-PSA-carrying glycopeptides and PSA-carrying glycopeptides, respectively. The values in parentheses indicate the ratios of the radioactivity of polysialylated *N*-glycans to that of non-polysialylated *N*-glycans in the preparations.

PSA-carrying glycoproteins prepared from	Radioactivity (cpm)	
	Non-polysialylated *N*-glycans	Polysialylated *N*-glycans
K1	17,000 (1.00)	27,800 (1.63)
Lec13	3,000 (1.00)	1,440 (0.48)
K1-II	15,240 (1.00)	29,600 (1.94)
Lec-II	6,410 (1.00)	4,360 (0.68)



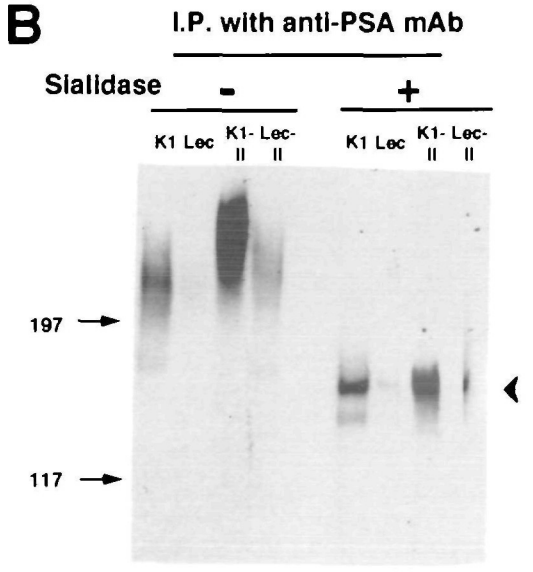


Fig. 2. **Metabolic labeling with [^{3}H]$GlcNH_2$ of NCAM and PSA-carrying glycoproteins in CHO cells.** Cells were labeled with [^{3}H]-$GlcNH_2$ for 48 h, and then lysed with a lysis buffer as described under "MATERIALS AND METHODS." PSA-carrying glycoproteins were isolated by immunoprecipitation with anti-PSA mAb. NCAM was isolated by immunoprecipitation with anti-NCAM mAb before and after the cell lysate had been treated with sialidase. The radioactivity in an aliquot of the resin was counted, and the rest of the resin was analyzed by SDS-PAGE on a 5-20% gradient gel before and after treatment with neuraminidase F (0.5 U/ml) for 3 h, followed by autoradiography. A: SDS-PAGE (5-20% gel) of NCAM and PSA-carrying glycoproteins from CHO K1 and Lec13 cells. B: SDS-PAGE (5% gel) of PSA-carrying glycoproteins from K1 and Lec13 cells, and their mST8Sia II cDNA-transfectants (K1-II and Lec-II) before and after sialidase treatment.

not the polysialylation in Lec13 cells was only different quantitatively from that in K1 cells. After sialidase treatment, PSA-carrying glycoproteins derived from both types of cells gave the same labeled band, the molecular weight of which was about 140 kDa (Fig. 2B). This 140 kDa glycoprotein could be re-immunoprecipitated with anti-NCAM mAb, indicating that NCAM-140 was polysialylated in both types of cells. To determine the proportion of PSA-carrying *N*-glycans in the PSA-carrying glycoproteins, glycopeptides prepared from PSA-carrying glycoproteins were separated by ion-exchange chromatography (Fig. 3). PSA-carrying glycopeptides obtained from Lec13 and K1 cells were eluted at almost the same salt concentration, suggesting that the degree of polymerization of α2,8-linked sialic acids in Lec13 was similar to that in K1 cells. However, the population of PSA-carrying *N*-glycans in the PSA-carrying glycoproteins from Lec13 cells was about 4-times less than that in the case of K1 cells (Table II). Even when glycopeptides were prepared from mST8Sia II cDNA-transfected Lec13 cells, the proportion of PSA-carrying glycopeptides was smaller than that in the case of K1 cells. In addition, PSA-carrying glycoproteins from mST8Sia II cDNA-transfected Lec13 cells migrated much faster than those from mST8Sia II cDNA-transfected K1 cells on SDS-PAGE (Fig. 2B). This suggests that the number of PSA-carrying *N*-linked oligosaccharides in Lec13 cells was less than that in K1 cells in the same PSA-carrying proteins. Therefore, polysialylation of the glycoproteins in Lec13 cells is different from that in K1 cells not only quantitatively but also qualitatively.

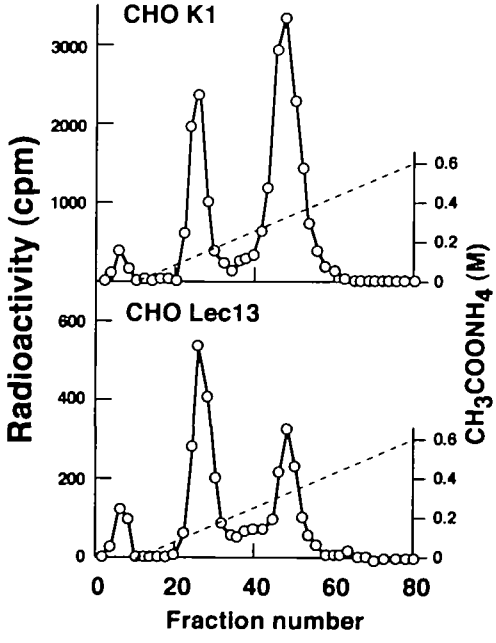


Fig. 3. **Ion-exchange chromatography of glycopeptides derived from PSA-carrying glycoproteins.** Cells were labeled with [^{3}H]$GlcNH_2$ for 48 h, and then lysed with a lysis buffer as described under "MATERIALS AND METHODS." PSA-carrying glycoproteins were isolated by immunoprecipitation with anti-PSA mAb. Glycopeptides were prepared by digestion with pronase K and separated on a DEAE-Toyopearl column, as described under "MATERIALS AND METHODS," and then subjected to anion-exchange chromatography on a DEAE-Toyopearl column with a linear gradient of 0-0.6 M CH_3COONH_4.

Comparison of N-Glycans Associated with NCAM—As described above, the major PSA-carrying glycoproteins in both types of cells are most probably NCAM-140. Since PSAs are usually carried on a branched chain attached to mannose through a β1,6-linked GlcNAc of tri- and tetra-antennary *N*-glycans associated with the 5th Ig-like domain of NCAM (*18, 19*), the differences in the antennary structures of *N*-glycans of NCAM may affect PSA synthesis and expression both quantitatively and qualitatively. Therefore, *N*-linked glycopeptides were isolated from NCAM preparations of K1 and Lec13 cells by digestion with pronase K, and the *N*-linked complex carbohydrates associated with NCAM were compared by lectin affinity chromatography. As shown in Fig. 4, about 90% of the *N*-glycans in NCAM preparations from both types of cells passed through a Con A-agarose column, and 10% of them was bound and eluted with 5 mM α-methyl mannoside, suggesting that the majority of *N*-glycans in the NCAM preparations from K1 and Lec13 cells had tri- or tetra-antennary structures, the rest of them having biantennary structures (*29*). The Con A-agarose unbound *N*-glycans of K1 and Lec13 cells were bound to a DSA-agarose column and eluted with 1% GlcNAc oligomer (Fig. 4), indicating that the *N*-glycans in the NCAM preparations from both types from cells contained β1,6-branched GlcNAc structures (*30*).

In the case of K1 cells, about 30% of the Con A-agarose unbound *N*-glycans was bound to LCA-agarose (Fig. 4), indicating that they are α1,6-fucosylated, β1,6-branched triantennary *N*-glycans (*31*). It is known that tri- or tetraantennary moieties containing branch GlcNAc residues linked β1,4 to mannose do not bind to LCA-agarose, even if they also contain a branch GlcNAc linked β1,6 to mannose and an α1,6-linked fucose. Therefore, the biantennary *N*-glycans (Con A-agarose bound *N*-glycans) of K1 cells were applied to a LCA-agarose column. Most of the biantennary *N*-glycans of K1 cells were bound to LCA-agarose and eluted with 0.2 M methyl-α-mannoside, indicating that most of the biantennary *N*-glycans of NCAM prepared from K1 cells contained α1,6-linked fucose residues. Judging from this result, most of the LCA-agarose unbound materials from K1 cells was probably tetra-antennary *N*-glycans modified with α1,6-fucose. In contrast, LCA-bound materials were not observed among either Con A-agarose bound or unbound glycopeptides derived from NCAM preparations from Lec13 cells, because Lec13 cells lack α1,6-fucose residues in their complex carbohydrates, as shown by Ripka *et al.* (*22*). The results of sequential lectin affinity chromatography of NCAM *N*-glycans indicated that there are no significant differences in the antennary structures of *N*-glycans between K1 and Lec13 cells except for α1,6-fucose modification, *i.e.*, NCAM of both types of cells contained tri- and tetraantennary complex carbohydrates with β1,6-GlcNAc branches as major *N*-linked oligosaccharides. Therefore, the absence of α1,6-linked fucose on complex *N*-glycans may affect the synthesis and/or expression of PSAs in Lec13 cells.

Effect of Fucose on the Expression of PSAs—To further confirm the possibility of the requirement of α1,6-linked fucose modification for PSA synthesis and expression, cells were first treated with endoneuraminidase, which specifically cleaves poly α2,8-sialic acids, and then the recovery

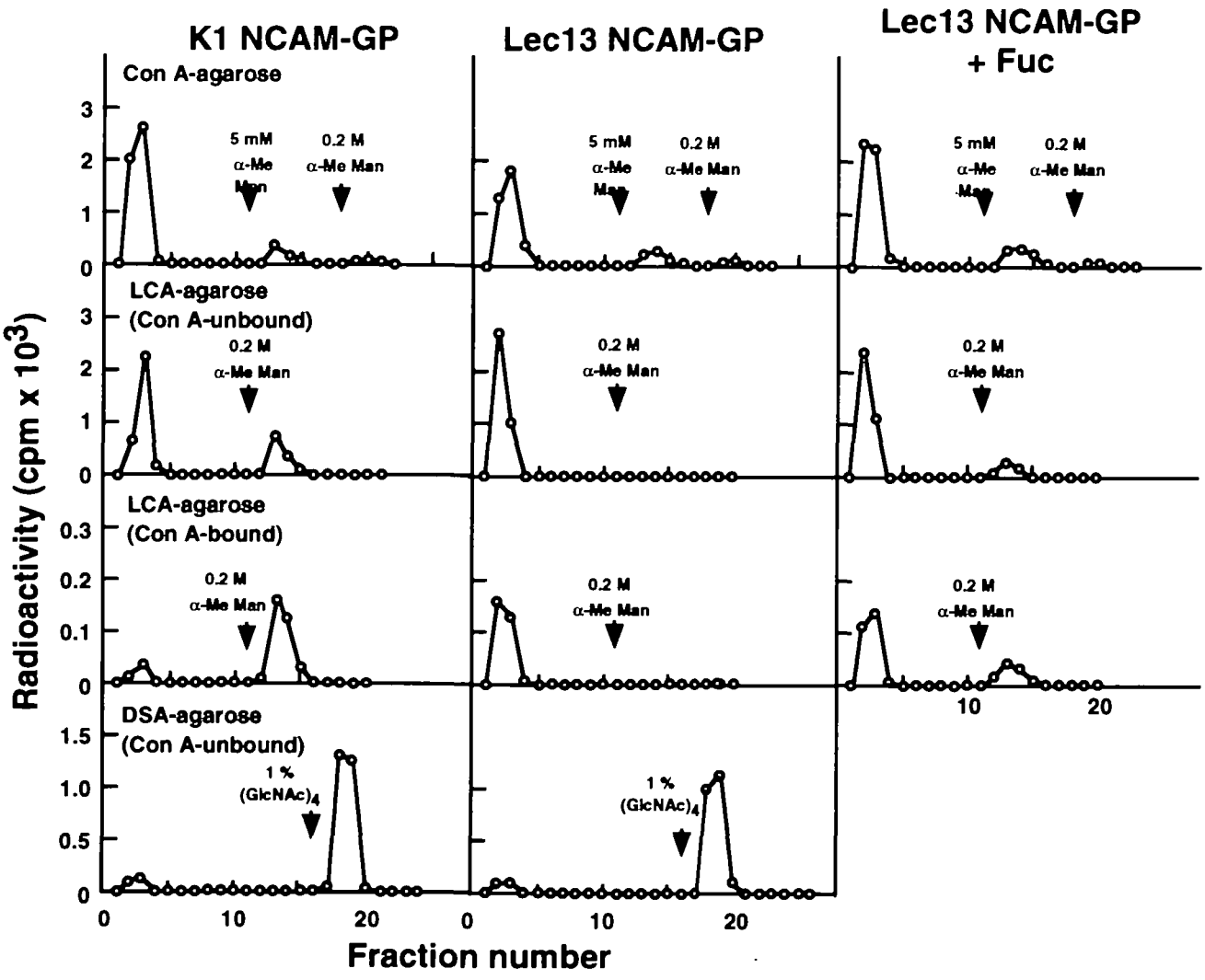


Fig. 4. **Lectin affinity chromatography of NCAM glycopeptides.** A labeled cell lysate was first treated with neuraminidase F and then NCAM was immunoprecipitated with anti NCAM mAb, followed by digestion with pronase K. The glycopeptides were subjected to lectin affinity chromatography under the conditions given under "MATERIALS AND METHODS." The glycopeptides were dissolved in 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% NaN_3, and then applied to a Con A-agarose column. The column was washed, and then eluted with 5 column volumes each of 5 mM methyl-α-mannoside and 0.2 M methyl-α-mannoside. Half of the Con A-agarose unbound materials was then applied to a DSA-agarose column. The column was washed and eluted with 1% *N*-acetylchitotetraose. The rest of the Con A-agarose unbound materials or Con A-agarose bound materials was applied to a LCA-agarose column. The column was washed and then eluted with 0.2 M methyl-α-mannoside. Fractions (2 ml) were collected and aliquots of 100 μl were counted with a liquid scintillation counter.

of PSA expression on the cell surface was determined with a fluorescence activated cell sorter (FACS). As shown in Fig. 5, PSAs on the cell surface were completely eliminated by the treatment with endoneuraminidase. In the case of CHO K1 cells, PSA expression on the cell surface gradually increased after endoneuraminidase treatment, about 80% of the PSAs being recovered after 72 h. In contrast, PSA expression increased much more slowly in Lec13 cells. Only 20% of the PSAs in Lec13 cells were recovered during the same period. This indicates that the rates of PSA synthesis and/or expression in K1 cells are much faster than those in fucose-lacking Lec13 cells. Lec13 cells are known to regain the lectin sensitivity on growth in medium containing L-fucose (*21*), indicating that fucose modification of complex carbohydrates occurs in Lec13 cells when the cells are grown in L-fucose containing medium. Therefore, the effect of exogenous L-fucose on PSA-expression was investigated. As shown in Fig. 6 and Table I, PSA expression and synthesis in Lec13 cells both increased about 2-fold when the cells were grown in L-fucose containing medium for 7 days, whereas those in K1 cells did not change.

To examine the occurrence of α1,6-fucosylation of *N*-glycans associated with NCAM after cultivation with L-fucose, glycopeptides were prepared from a NCAM preparation from Lec13 cells grown with 2 mM L-fucose, and then separated by sequential lectin affinity chromatography (Fig. 4). The ratio of Con A-bound and -unbound materials in the cells was not different from that in the case of Lec13 cells cultured without fucose. When the materials were applied to a LCA-agarose column, about 20% of the Con A-bound materials and 5% of the Con A-unbound

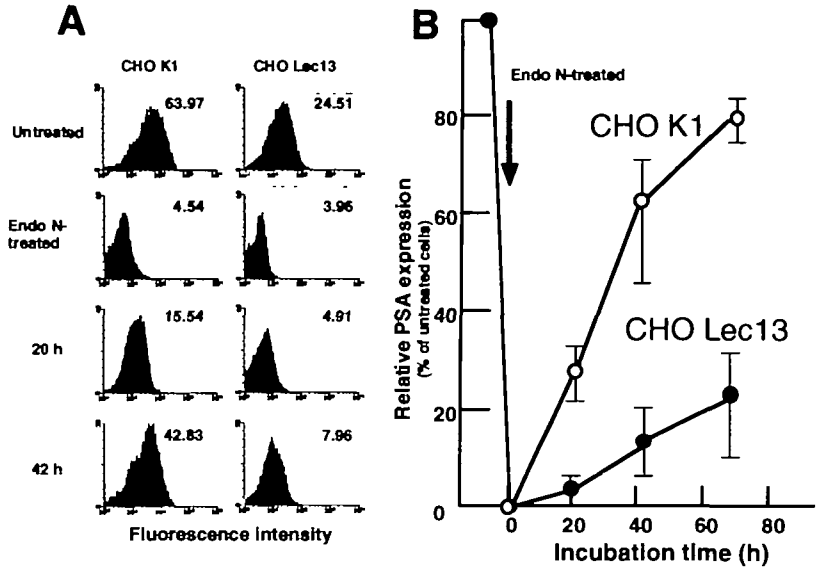


Fig. 5. **Recovery of PSA expression after endoneuraminidase treatment.** Cells were harvested and then treated with endoneuraminidase (1:10 dilution) for 1 h at 37°C. After the treatment, the cells were washed with PBS, and then one-fifth of the cells was fixed with 4% paraformaldehyde for 1 h at 4°C, washed with PBS, and stored at 4°C until analysis. 1×10^6 of the remaining cells were seeded into a 6-cm-diameter culture dish and cultured for 15, 40, 60, or 72 h. After the incubation, the cells were harvested using an EDTA/PBS solution and then fixed with 4% paraformaldehyde. The fixed cells were treated with 1% BSA-containing PBS for 1 h at 4°C, and then stained with anti-PSA-mAb or nonimmunized mouse IgG, followed by analysis of PSA expression with a flow cytometer. A: Typical FACS pattern of PSA expression after endoneuraminidase treatment. B: Recovery of PSA expression. Each value represents the mean fluorescence intensity. Bars represent the means±SD for four independent experiments. Open and closed circles indicate CHO K1 and Lec13 cells, respectively.

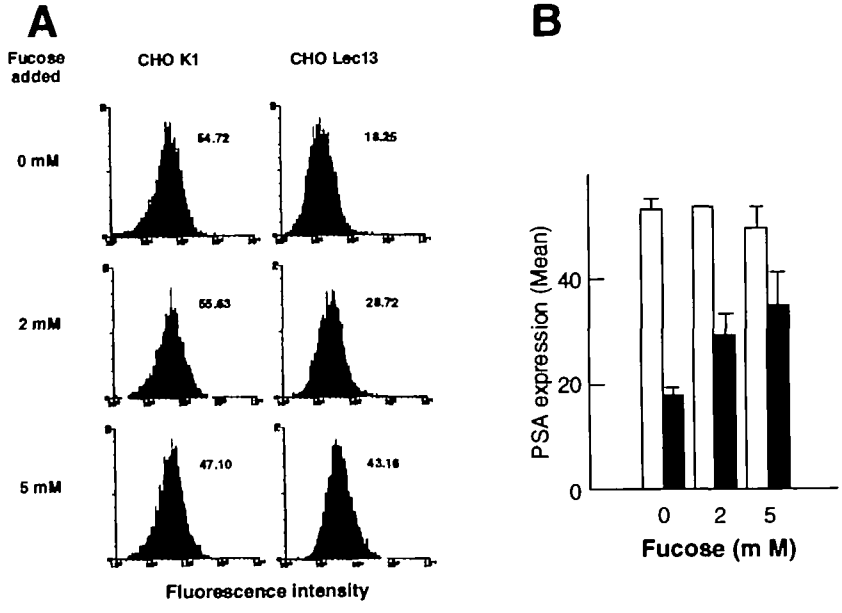


Fig. 6. **Effect of fucose on the expression of PSAs.** Cells were maintained in 1, 2, or 5 mM L-fucose containing alpha medium for 7 days. The medium was changed every 2 days. The expression of PSA was measured with a FACS. A: Typical FACS pattern of PSA expression after growth with fucose. B: Expression of PSAs after growth with fucose based on the mean fluorescence intensity values in panel A. Each value represents the mean fluorescence intensity. Bars represent the means±SD for four independent experiments. Open and solid columns indicate CHO K1 and Lec13 cells, respectively.

materials bound to the column and were eluted with 0.2 M methyl-α-mannoside, although no LCA-agarose binding materials were observed in the NCAM preparation from Lec13 cells grown without L-fucose. These results indicated that at least a part of the *N*-glycans of NCAM prepared from Lec13 cells was α1,6-fucosylated after the cells had been cultured with L-fucose.

PSA-Carrying Proteins Are Degraded in Fucose-Lacking Mutant Lec13 Cells—Angata *et al.* reported that α1,6-linked fucose lacking NCAM-Fc produced by Lec13 cells was polysialylated by recombinant PSA-synthases as equally as α1,6-linked fucose containing NCAM-Fc produced by K1 cells (*23*). To determine whether or not fucose modification affects the biosynthesis of PSA or the expression of PSA *in vivo*, cells were pulse-labeled with [^{35}S]Met for 10 min, followed by a chase for 1, 2, 4, or 8 h. After the pulse-chasing of the cells, PSA-carrying proteins were recovered by immunoprecipitation with anti-PSA mAb, and then analyzed by SDS-PAGE. As shown in Fig. 7, both K1 and Lec13 cells synthesized PSA-carrying proteins within a 1 h chase after a 10 min pulse. Judging from the radioactivity recovered on immunoprecipitation with anti-PSA mAb, PSA synthesis in Lec13 cells within a 1 h chase seemed to be only 2 times lower than that in K1 cells. This difference was not significant as compared to the difference in metabolic labeling of the cells with [^{3}H]$GlcNH_2$ for 48 h. However, the radioactivity recovered in PSA-carrying proteins of Lec13 cells gradually decreased during a prolonged chase period. After a 8 h chase, only 20% of the PSA-carrying proteins remained as compared to those obtained from cells after a 1 h chase (Fig. 7). In this process, PSA-carrying proteins were degraded into a polypeptide with a molecular weight of about 85 kDa and several other polypeptides. In contrast, the radioactivity recovered in PSA-carrying proteins in K1 cells did not change during a 8 h chase. The degradative polypeptide (85

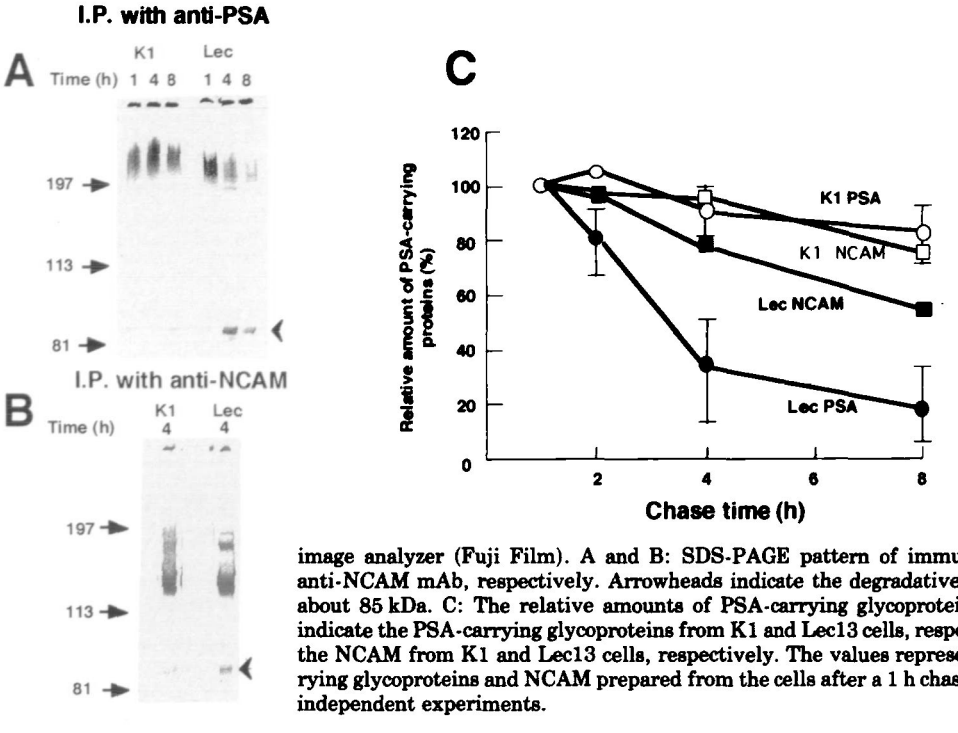


Fig. 7. **Pulse-chase labeling of PSA-carrying glycoproteins.** Cells were pulse-labeled with [^{35}S]Met (100 μCi/ml) in methionine-free DMEM for 10 min at 37°C, and then chased for 1, 2, 4, or 8 h, as described under "MATERIALS AND METHODS." Each cell lysate was precleared with nonimmunized mouse IgG and protein G-Sepharose for 16 h at 4°C with gentle rotation, and then the resulting supernatant was immunoprecipitated with anti-NCAM mAb or anti-PSA mAb, and protein G-Sepharose, as described under "MATERIALS AND METHODS." The resin was collected by centrifugation and then subjected to SDS-PAGE on a 5% acrylamide gel before and after treatment with neuraminidase F. The gels were fixed and dried, and radioactivity was detected and counted with a BAS2000 radio image analyzer (Fuji Film). A and B: SDS-PAGE pattern of immunoprecipitates with anti-PSA mAb and anti-NCAM mAb, respectively. Arrowheads indicate the degradative polypeptide with a molecular weight of about 85 kDa. C: The relative amounts of PSA-carrying glycoproteins and NCAM. Open and closed circles indicate the PSA-carrying glycoproteins from K1 and Lec13 cells, respectively. Open and closed squares indicate the NCAM from K1 and Lec13 cells, respectively. The values represent the relative radioactivity of PSA-carrying glycoproteins and NCAM prepared from the cells after a 1 h chase. Bars represent the means ± SD for four independent experiments.

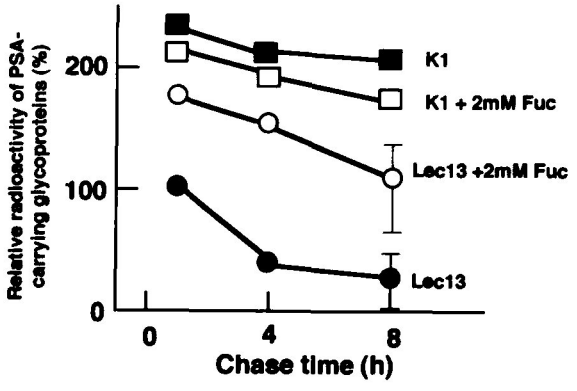


Fig. 8. **Effect of fucose on the synthesis of PSA-carrying glycoproteins.** Cells were cultured with or without 2 mM L-fucose containing alpha medium for 3 days, pulse-labeled with [^{35}S]Met (100 μCi/ml) in methionine-free DMEM for 10 min at 37°C, and then chased for 1, 4, or 8 h, as described under "MATERIALS AND METHODS." PSA-carrying glycoproteins were recovered by immunoprecipitation with anti-PSA mAb, and then subjected to SDS-PAGE on a 5% acrylamide gel. Each gel was fixed and dried, and radioactivity was detected and counted with a BAS2000 radio image analyzer (Fuji Film). Open and closed circles indicate the PSA-carrying glycoproteins from Lec13 cells cultured with and without 2 mM fucose, respectively. Open and closed squares indicate the PSA-carrying glycoproteins from K1 cells cultured with and without 2 mM fucose, respectively. Bars represent the means ± SD for three experiments.

kDa polypeptide) was not observed at all during a 4 h chase, but a small amount of it could be detected after a 8 h chase even in K1 cells (Fig. 7). On the other hand, the radioactivity in NCAM preparations from the two types of cells was not significantly different. In the NCAM preparation prepared from cells after a 4 h chase, the degradative 85 kDa polypeptide was clearly observed but polydisperse material could not be detected in Lec13 cells, suggesting that this 85 kDa polypeptide was derived from polysialylated NCAM. These results suggested that PSA-carrying proteins in Lec13 cells were rapidly degraded after PSA modification of the proteins. Since PSA-carrying proteins disappeared during the chase, the ratio of the radioactivity recovered in PSA-carrying proteins in Lec13 and K1 after a 8 h chase was about 1:10, which was a similar value to that obtained on metabolic labeling with [^{3}H]GlcNH$_2$ for 48 h. PSA-carrying proteins were rapidly degraded even in mST8Sia II cDNA-transfected Lec13 cells (data not shown).

When Lec13 cells were cultured in 2 mM L-fucose containing medium for 48 h before the pulse-chase experiment, the radioactivity recovered in PSA-carrying materials in Lec13 cells during a 1 h chase was about 2 times that in control cells cultured without fucose (Fig. 8). In addition, the degradation of the PSA-carrying materials during a prolonged chase period was inhibited in the cells cultured with fucose (Fig. 8). In contrast, the synthesis of PSA-carrying proteins in K1 cells cultured with fucose was slightly inhibited. These results suggested that the α1,6-fucosylation of *N*-glycans promotes PSA synthesis and prevents the degradation of synthesized PSA-carrying proteins. Thus, the lower and slower expression of PSA in fucose-lacking Lec13 cells may be explained primarily by the instability of the synthesized PSA in the cells, and secondly by the low polysialylation efficiency.

DISCUSSION

Since it was recently shown that PSA-carrying *N*-glycans are almost completely α1,6-fucosylated at the proximal GlcNAc of the di-*N*-acetyl chitobiose unit (*19*), and α1,6-

fucosylated glycoproteins are predominantly polysialylated by recombinant polysialic acid synthases *in vitro* (*9*), the α1,6-linked fucose modification of *N*-glycans associated with NCAM may act as a signal for PSA synthesis. In this study, therefore, to determine the role of α1,6-linked fucose in the expression of PSAs, we compared PSA synthesis and expression between the CHO cell line, K1, and the fucose-lacking CHO mutant line, Lec13. Lec13 cells were originally established as a pea-lectin resistant CHO mutant (*21*), and shown to lack α1,6-linked fucose attached to the proximal GlcNAc of the di-*N*-acetyl chitobiose unit of *N*-glycans of all glycoproteins (*22*). As shown in this study, α1,6-linked fucose modification of *N*-glycans was not essential for the biosynthesis of PSAs but was required for the efficient expression of PSA-carrying glycoproteins on the surface of CHO cells. The following results strongly suggest that the lack of α1,6-linked fucose on *N*-glycans reduced the stability of PSA-carrying glycoproteins in CHO cells; (i) Lec13 cells express PSAs on their surface like K1 cells, but the level of expression of PSAs in Lec13 cells was lower than that in K1 cells; (ii) the incorporation of labeled glucosamine into PSA-carrying glycoproteins in Lec13 cells was over 10-times lower than that in K1 cells, although that into NCAM as well as total extracts of Lec13 and K1 cells was almost the same; (iii) when Lec13 cells were cultured in fucose-containing medium, in which Lec13 cells are known to regain the sensitivity toward pea lectin, PSA expression clearly increased; and (iv) a pulse-chase experiment with [^{35}S]Met revealed that PSA-carrying proteins in Lec13 cells gradually decreased during a prolonged chase period, and that the degradation of PSA-carrying proteins was partly prevented by cultivation of the cells with fucose.

The expression of PSAs in Lec13 cells is remarkably lower and slower than that in K1 cells. The differences in the expression and biosynthesis of PSAs between Lec13 and K1 cells might be simply explained as due to differences in PSA synthase activity, the intracellular amount of NCAM, and/or the antennary structures of complex *N*-glycans of NCAM, but the following results should exclude such possibilities. In CHO cells, ST8Sia IV, of the two kinds of PSA synthase, has been shown to be the only factor necessary for PSA synthesis and expression (*3, 20*). In the case of Lec13 cells, ST8Sia IV but not ST8Sia II is expressed (data not shown), and the PSA synthase activity in the two types of cells is not significantly different. Even if PSA synthase was overexpressed in Lec13 cells on transfection with the cDNA encoding mST8Sia II, the *de novo* PSA synthesis and expression were still lower than those in the cDNA non-transfected K1 cells (Fig. 2B). In addition, the α2,3- and α2,6-sialyltransferase activities in Lec13 cells were almost the same as those in K1 cells. Metabolic labeling of cells suggested that NCAM expression was not significantly different between the two types of cells (Fig. 2A). The same glycoprotein, whose molecular mass is about 140 kDa, is polysialylated in the two types of cells. This 140 kDa glycoprotein is most probably NCAM-140, because the 140 kDa glycoprotein could be immunoprecipitated with anti-NCAM mAb from the immunoprecipitates with anti-PSA mAb after sialidase treatment. Furthermore, most of the *N*-glycans of NCAM in both types of cells had tri- and tetraantennary structures with a branch GlcNAc linked β1,6 to mannose (Fig. 4), based on the carbohydrate-binding specificities of Con A and DSA (*29, 30*).

In contrast, *N*-glycans associated with NCAM prepared from Lec13 cells did not contain α1,6-linked fucose, while *N*-glycans associated with NCAM in K1 cells are thought to contain α1,6-linked fucose residues, judging from the results of LCA-agarose affinity chromatography of glycopeptides (*31*). Since there are no significant differences in the levels of NCAM synthesis and PSA synthase activity, or the antennary structures of *N*-glycans associated with NCAM between the two types of cells, the absence of α1,6-linked fucose residues on *N*-glycans associated with NCAM is considered to be the only difference as to the PSA synthesis and expression of Lec13 from K1 cells. Therefore, the absence of α1,6-linked fucose residues is assumed to be one of the causes of the lower and slower PSA synthesis and expression in Lec13 cells. This assumption was supported by the increased expression of PSA in cells cultured with L-fucose. It is known that Lec13 cells regained the sensitivity toward pea lectin, strongly recognizing α1,6-linked fucose residues (*31*), on growth in fucose-containing medium (*21*). A part of the *N*-glycans of NCAM was α1,6-fucosylated after Lec13 cells had been grown in fucose-containing medium (Fig. 4). On the other hand, blood group H, Le^x, and Le^y were not expressed on Lec13 cells even after cultivation with fucose. CHO cells have well defined glycans on their glycoproteins and glycolipids, and do not synthesize Fucα1,2Gal and Fucα1,3/4GlcNAc linkages (*32, 33*). Therefore, only α1,6-linked fucose modification would occur in Lec13 cells cultured with fucose.

The α1,6-fucosylation of *N*-glycans probably affects the PSA synthesis process, because *in vivo* PSA synthesis in the two types of cells differs qualitatively as well as quantitatively (Fig. 3 and Table II). However, the lower and slower PSA expression in Lec13 cells can be mainly explained by the instability of PSA-carrying glycoproteins rather than the slower rate of PSA synthesis in Lec13 cells. It was surprising that in Lec13 cells PSA-carrying proteins were rapidly (within 8 h) degraded after PSAs had been synthesized (Fig. 7). On the other hand, the degradation of PSA-carrying proteins in K1 cells was not observed at all during the chase period. The degradation of PSA-carrying proteins in Lec13 cells was partly prevented when the cells were grown in fucose-containing medium. These results suggest that α1,6-fucosylation of *N*-glycans in PSA-carrying glycoproteins may play a role in the post-translational stability of the PSA-carrying glycoproteins in CHO cells.

It was shown that highly purified polysialylated NCAM was automatically degraded completely in a low salt buffer at 37°C for 96 h (*34*). This was only observed *in vitro* and was assumed to be the result of intrinsic proteolytic activity or a contaminating tightly bound enzyme. Therefore, the possibility that the specific proteolytic activity is only expressed in Lec13 cells, *i.e.* not K1 cells, can not be completely excluded. However, even in K1 cells, a prolonged chase produced the same degradative polypeptide (Fig. 7). Based on the spontaneous proteolysis of polysialylated NCAM *in vitro*, it was suggested that there is at least one compact proteinase-sensitive domain in the polypeptide chain (*34*). Therefore, the α1,6-fucosylation of *N*-glycans may prevent the proteinase-sensitive domain of *N*-glycans undergoing a conformational change. If there is proteolytic activity tightly bound to or closely associated with NCAM, it may be possible that the proteinase

selectively cleaves non α1,6-fucosylated NCAM, and thereby the PSA-carrying *N*-glycans of NCAM are almost completely fucosylated on the proximal GlcNAc, as shown in chick brain (*19*). Alternatively, de-*N*-glycosylation of the nonfucosylated *N*-glycans of NCAM by PNGase may trigger the degradation of PSA-NCAM, because the activity of PNGase purified from L-929 mouse fibroblast cells was shown to be inhibited in the presence of α1,6-linked fucose, and PNGase activity could be detected ubiquitously in mammalian cells (*35*). It was recently shown that the quality of ovalbumin was controlled through site-specific de-*N*-glycosylation by PNGase (*36*). It is not clear at this time whether the stability of only PSA-carrying glycoproteins is affected by fucose modification or those of other glycoproteins are also affected. Judging from the results of metabolic labeling with [^{3}H]GlcNH$_2$ for 48 h, there was no significant difference in the SDS-PAGE patterns between total extracts of Lec13 and K1 cells, suggesting that the expression of the major glycoproteins in CHO cells may not be affected by fucose modification. In addition, the incorporation of labeled glucosamine into NCAM during the labeling period was almost the same in the two types of cells. Judging from the results of a pulse-chase experiment involving [^{35}S]Met, newly synthesized NCAM in Lec13 cells seemed to be more rapidly degraded than that in K1 cells (Fig. 7). Therefore, it is possible that α1,6-linked fucose plays a role in the expression of not only the polysialylated form of NCAM but also other glycoproteins through its stability in CHO cells.

Although α1,6-linked fucose residues attached to the proximal GlcNAc of *N*-glycans have been shown to have some relationship with carcinogenesis (*37-39*), only little is known about their function. It has been reported that fucose-containing glycoproteins are cleared rapidly from the blood into the liver (*40*), and that nonfucosylated complex *N*-glycans are more stable than fucosylated *N*-glycans as to the action of an endo-β-*N*-acetylglucosaminidase F/PNGase mixture (*41*). On the other hand, the activity of PNGase purified from mammalian cells is inhibited in the presence of α1,6-linked fucose (*35*). Therefore, α1,6-linked fucose may contribute to the quality control of glycoproteins. Here, we presumed a novel function of α1,6-linked fucose of *N*-glycans, *i.e.*, α1,6-linked fucose modification of *N*-glycans affects the expression of certain glycoproteins (*e.g.* NCAM) through the intracellular stability of the glycoproteins and the quality of polysialylation *in vivo*. Recently, cDNA encoding an α1,6-fucosyltransferase was cloned from porcine and human sources (*42, 43*). Since α1,6-fucosyltransferase mRNA is most abundantly expressed in the brains of several mammals (*42*), and α1,6-linked fucose of *N*-glycans is encountered frequently in brain glycoproteins (*44*), manipulation of the α1,6-fucosyltransferase gene is required for elucidation of the function of α1,6-linked fucose in brain glycoproteins.

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