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# Core saccharide dependence of sialyl Lewis X biosynthesis

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**Abstract** The sialyl-Lewis X ($SLe^x$) determinant is important in leukocyte extravasation, metastasis and bacterial adhesion. The role of the protein, N-glycan and O-glycan core structures for the biosynthesis of $SLe^x$ *in vivo* by fucosyltransferases (FucTs) is not known. Immunoglobulin G (IgG) Fc fusion proteins of $\alpha_1$-acid glycoprotein (AGP), P-selectin glycoprotein ligand-1 (PSGL-1) or CD43 were used to probe the specificity of FucT-III-VII expressed alone in 293T and COS cells or together with O-glycan core enzymes in Chinese hamster ovary (CHO)-K1 cells. Western blotting with the monoclonal antibodies CSLEX and KM93 showed that FucT-III and V-VII produced $SLe^x$ on core 2 in CHO cells. Only FucT-V, -VI and, with low activity, -VII worked on core 3 on CD43/IgG, but no $SLe^x$ was detected with CSLEX on PSGL-1/IgG with core 3. KM93 stained $SLe^x$ on core 2, but was not reactive with $SLe^x$ on core 3. FucT-III, V-VII made $SLe^x$ on N-glycans of AGP/IgG in CHO, but not in COS and 293T cells, even though the same FucTs could make $SLe^x$ on CD43/IgG and PSGL-1/IgG in these cells. Our results define the specificities of FucT-III-VII in $SLe^x$ biosynthesis on O-glycans with different core structures and the fine specificity of the widely used anti-$SLe^x$ monoclonal antibody, KM93.

**Keywords** Sialyl Lewis X · α3-fucosyltransferase · Cancer-associated epitopes · O-glycan biosynthesis

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## Introduction

The carbohydrate determinant sialyl-Lewis X ($SLe^x$) is important in a wide variety of biological processes, such as bacterial infection [1, 2], leukocyte extravasation [3–5] metastasis and cancer [6–12]. It is known that the carrier protein and the type of glycan and core saccharide carrying $SLe^x$ are essential for P-selectin binding. P-selectin glycoprotein ligand-1 (PSGL-1) modified by $SLe^x$ can bind to all the selectins [3, 13]. However, for other lectins binding $SLe^x$, the role of the glycoconjugate for correct presentation of $SLe^x$ is not clear.

$SLe^x$ is formed by fucosylation of its precursor 3'sialyl-lactosamine, Siaα3Galβ4GlcNAc. Five fucosyltransferases (FucTs), FucT-III-VII, have been cloned that can make $SLe^x$ in man [14]. Their *in vitro* specificities have been extensively studied [reviewed in 14], but only a few investigations have evaluated their *in vivo* specificities. One paper describes the $SLe^x$-biosynthesis *in vivo* on β-trace glycoprotein that carries only N-glycans [15], and two papers describe its biosynthesis on glycolipids [16, 17]. Reports so far on FucT specificity on O-glycans *in vivo* have usually been focused on FucT-IV and FucT-VII [18–21]. All these studies point to differences in substrate specificity of the α3FucTs. Knowing more in detail about their specificities *in vivo* is necessary to be able to define the biological role of the individual FucTs. It also provides a mean to investigate the biology of tumor metastasis as well as bacterial adhesion better, and thereby also facilitate the development of novel therapeutics.

O-glycans have different core structures and the most common ones in humans are core 1–4 [22, 23]. Although the importance of core 2 in selectin binding has been shown in many publications [24–27], the role of $SLe^x$ presented on the core 3 structure is not clear. The core saccharide

carrying SLe$^x$ may influence its binding to selectins, which might prove important in metastasis [8, 20, 28]. In fact, Mitoma *et al*. [20] found evidence for a difference in binding of selectins between PSGL-1 carrying SLe$^x$ on extended core 1 and core 2. A recent study suggested that a high metastatic capacity of colon cancer cells correlated with down-regulation of the core 3 synthase, β3-*N*-acetylglycosamine transferase VI (β3GlcNAcT-VI, [29]). However, the mechanism behind this remains unknown.

To address the role of core O-glycan structures for FucT specificity, as well as studying the role of the different α3FucTs in the *in vivo* biosynthesis of SLe$^x$ on both N- and O-glycans, we transiently expressed FucT-III-VII in 293T, COS-7m6 and Chinese hamster ovary (CHO)-K1 cells. In CHO cells, which are known to have only core 1 O-glycans [30], we also expressed core 2 β6GlcNAcT-I (C2GnT-I, [31]) or core 3 β3GlcNAcT-VI. In addition, immunoglobulin G (IgG) Fc fusion proteins of $\alpha_1$-acid glycoprotein (AGP), PSGL-1 and CD43 were expressed as probes for fucosyltransferase specificity. AGP carries only N-glycans, PSGL-1 mainly O-glycans and CD43 only O-glycans [32]. On CD43/IgG, FucT-III, -V, -VI and -VII gave rise to SLe$^x$ on core 2 on O-glycans in CHO-cells, whereas only FucT V, VI and, to a certain extent, -VII were effective in producing SLe$^x$ on core 3. PSGL-1 was also accepted as carrier protein for SLe$^x$ production by FucT-III, -V, -VI and -VII on core 2. On the other hand, SLe$^x$ on core 3 could not be detected at all on PSGL-1. The monoclonal antibody (mAb) KM93 reacted well with SLe$^x$ on core 2, but did not at all stain the same epitope on core 3 O-glycans regardless of the fusion protein used as probe. FucT-III, -V, -VI and -VII were all able to produce SLe$^x$ on AGP in CHO, but not in COS and 293T cells, even though these FucTs could make SLe$^x$ on CD43/IgG and PSGL-1/IgG in the same cells.

## Materials and methods

### Plasmid construction

See Table 1 for primer sequences.

**Table 1** Primers used for PCR amplification and subcloning of cDNAs used in the expression vectors

| | cDNA | Forward | Reverse | Internal forward | Internal reverse |
|---|---|---|---|---|---|
| Fusion proteins | CD43 | CGC GGG AAG CTT ACC ATG GCC ACG CTT CTC CTT | CGC GGG GCG GCC TTA AGG GGC AGC CCC GTC | | |
| | AGP | CGC GGG GCT AGC CCA GAT CCC ATT GTG TG | CGC GGG GGA TCC GAT TCC CCC TCC TCC TG | | |
| | PSGL-1 | [33] | | | |
| Core enzymes | β6GlcNAcT-I (core 2) | [30] | | | |
| | β3GlcNAcT-VI (core 3) | CGC GGG AAG CTT ACC ATG GCT TTT CCC TGC CGC | CGC GGG TCT AGA TCA GGA GAC CCG GTG TCC | | |
| α1,3/4Fuc-Ts | FucT-III | CGC GGG CTC GAG GCC GCC ACC ATG GAT CCC CTG GGT GCA GCC AAG | CGC GGG GCG GCC GCT CAG GTG AAC CAA GCC GCT ATG CTG | | |
| | FucT-IV | cDNA expression clone | | | |
| | FucT-VI | CGC GGG AAG CTT ACC ATG GAT CCC CTG GGC CCA GCC AAG | CGC GGG GCG GCC GCT CAG GTG AAC CAA GCC GCT ATG CTG | GGA GTC CCC CAG CAA CTG CCG | CCA GGG CTT CCA GGT GCC GGC AGT TGC T |
| | FucT-VI | CGC GGG AAG CTT ACC ATG GAT CCC CTG GGC CCA GCC AAG | CGC GGG GCG GCC GCT CAG GTG AAC CAA GCC GCT ATG CTG | AGC CAC TGC TGG CAG CTG AAA GCC A | GAA GTA TCC GTC CAT GGC TTT CAG CTG CCA |
| | FucT-VII | CGC GGG AAG CTT ACC ATG AAT AAT GCT GGG CAC GGC | CGC GGG TCT AGA TTT AGG CCT GAA ACC AAC CCT CAA G | | |

*Fusion proteins* The cDNA encoding the extracellular part of CD43 was amplified by PCR from an expression plasmid encoding full length CD43 (a kind gift of Prof. Brian Seed, Dept. of Molecular Biology, MGH, Boston, MA, USA), and subcloned into the mouse $IgG_{2b}$ Fc expression cassette using *Hind*III and *Bam*H1. Similarly, the $\alpha_1$-acid glycoprotein (AGP)-coding sequence was PCR amplified, excluding the stop codon and the leader peptide, from a human liver cDNA library. The AGP cDNA was fused in frame with the cDNA encoding the CD5 leader sequence upstream and the Fc portion of mouse $IgG_{2b}$ downstream using the *Nhe*I and *Bam*HI sites in the expression cassette. The PSGL-1/mIgG plasmid was constructed as described [33].The same vector backbone was used for all fusion protein constructs.

*Core enzymes* The C2GnT-I cDNA was constructed as described before [30]. The core 3 synthase was PCR amplified from human stomach cDNA and subcloned into CDM8 using *Hind*III and *Xba*I.

*α3FucTs* The Lewis gene (FUT3) encoding FucT expression plasmid was a kind gift of Prof. Brian Seed. FUT4 was isolated as a cDNA clone from an HL60 cDNA expression library (Seed, B. *et al*., unpublished). FUT5 was amplified in one amino- and one carboxy-terminal fragment by PCR using placental genomic DNA as template and internal overlapping primers containing a *Nae*I site. The two pieces were cloned into a modified CDM8 vector having *Nae*I in the polylinker, but lacking the M13 origin of replication and its *Nae*I site. Similarly, FUT6 was also amplified from placental genomic DNA in two pieces. The two pieces were subcloned and linked together in a shuttle vector and then moved in one piece into CDM8 using *Hind*III and *Not*I. FUT7 was amplified from the HL60 cDNA library and was subcloned into CDM8 using *Hind*III and *Xba*I.

## Albumin conjugates

$SLe^x$ and 3'sialyllactosamine bovine serum albumin (BSA) conjugates were purchased from Dextra Laboratories, Reading, UK.

## Transfections and cell culture

Recombinant proteins were produced by transient transfections of 293T and COS-7m6 cells as before [34]. CHO cells were transfected in 25 $cm^2$ T-flasks (BD Falcon, Bedford, MA, USA) with 20 μl of Lipofectamine 2000 (Invitrogen, Paisley, UK) according the manufacturer's instructions. Plasmids encoding protein/IgG, glycosyltransferases and, in some cases, empty vector, were used in a total amount of 10 μg. Cells were cultured and supernatants harvested as described [34].

## Antibodies

All antibodies were diluted in 3% BSA in phosphate buffered saline (PBS) with 0.05% Tween 20 (PBST) or

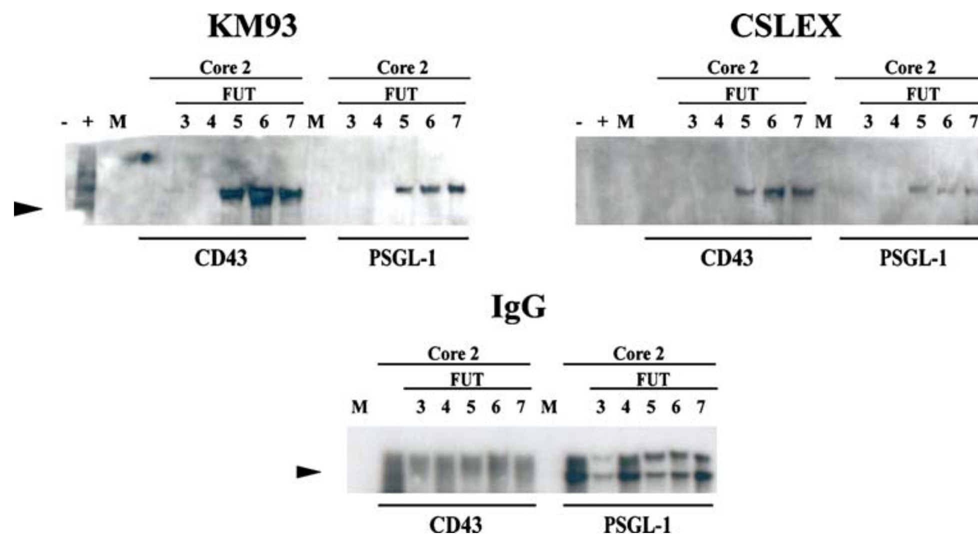


**Fig. 1** SDS-PAGE and Western blot analysis of immuno-purified CD43/- and PSGL-1/$mIgG_{2b}$ proteins produced in CHO-K1 cells transfected with or without β6GlcNAcT-I (core 2), in combination with different genes encoding FucTs. Following separation under non-reducing conditions on an 4–12% SDS-PAGE and blotting onto nitrocellulose membranes, the recombinant proteins were probed with two separate anti-$SLe^x$ antibodies (KM93, left panel, and CSLEX, right panel), followed by an HRP conjugated secondary antibody. In all lanes, similar amounts of fusion protein were loaded as shown by the anti-mIgG antibody reactivity (*lower panel*). The *arrow* indicates 191 kDa and samples from mock-transfected cells are indicated by *M*. *Positive* and *negative signs* denote BSA conjugated to $SLe^x$ and 3'sialyllactosamine, respectively

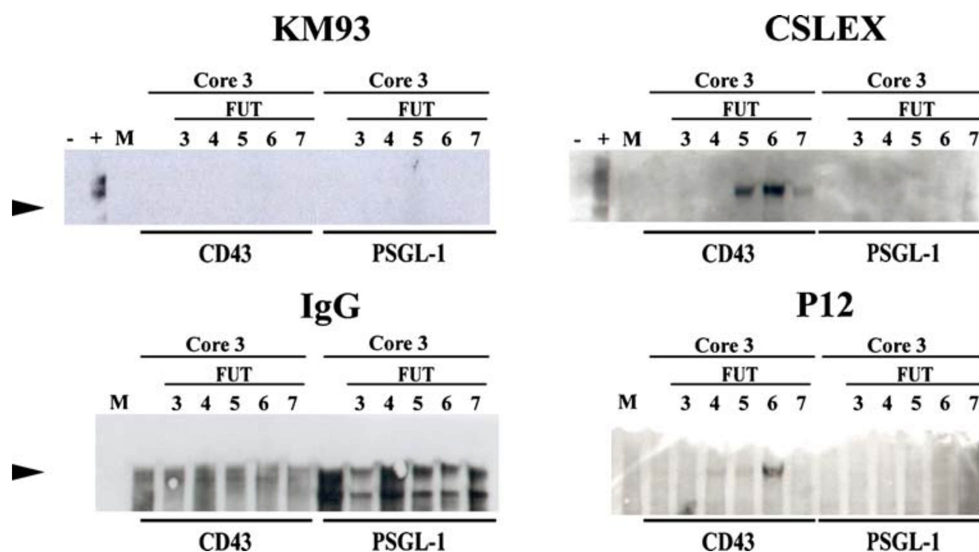


**Fig. 2** SDS-PAGE and Western blot analysis of immuno-purified CD43/- and PSGL-1/mIgG$_{2b}$ proteins produced in CHO-K1 cells transfected with or without β3GlcNAcT-VI (core 3), in combination with different genes encoding FucTs. Recombinant proteins were separated under non-reducing conditions on a 4–12% SDS-PAGE and blotted onto nitrocellulose membranes. They were probed with anti-SLe$^x$ antibodies (KM93, *upper left panel*, and CSLEX, *upper right panel*), or an anti-Le$^x$ antibody (P12, *lower right panel*) followed by an HRP conjugated secondary antibody. In all lanes, similar amounts of fusion protein were loaded as shown by the anti-mIgG antibody reactivity (*lower left panel*). The arrow indicates 191 kDa and samples from mock-transfected cells are indicated by *M*. *Positive* and *negative signs* denote BSA conjugated to SLe$^x$ and 3'sialyllactosamine, respectively

only PBST. Horseradish peroxidase-conjugated goat anti-mouse IgM (Pierce, Rockford, IL, USA) was used at a dilution of 1:80,000–160,000. KM93 (Calbiochem, Merck, Darmstadt, Germany) was used at a dilution of 1:200. Culture of CSLEX-1 hybridoma cells (ATCC) and purification of antibodies were as in [34]. Purified antibodies were diluted to 1 μg/μl and used at a dilution of 1:200.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Recombinant proteins were purified as before [34]. After immunoprecipitation and washing twice in PBS, the agarose beads were mixed with 50 μl of 2× lithium dodecyl sulfate-sample buffer (Invitrogen) and heated at 70°C for 10 min. Samples, 10 μl, were loaded on a 4–12% NUPAGE-gel (Invitrogen). Electrophoresis was run at 200 V, for about 60 min. The samples were subsequently blotted onto 0.2 μm nitrocellulose membranes (Invitrogen) at 100 V between 60 and 90 min in a Mini Protean II transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% BSA/PBST overnight at 4°C. Washing steps in between all incubations were performed with an initial quick rinse using washing buffer followed by three changes of buffer, 5 min for each change. Thereafter, membranes were developed as before [34]. Shown are representative results from one of three independent experiments.

## Results

### O-glycans

The biosynthesis of SLe$^x$ on O-glycans expressed in CHO cells differed between core 2 and 3. As expected, without

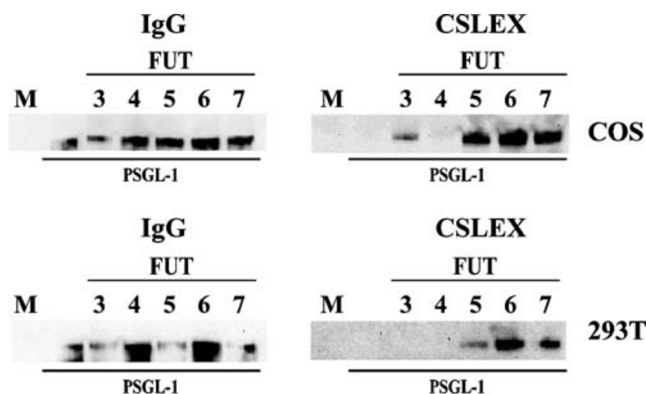


**Fig. 3** SDS-PAGE and Western blot analysis of immuno-purified PSGL-1/mIgG$_{2b}$ proteins produced in 293T and COS7-m6 cells transfected with or without different genes encoding FucTs. Following separation under non-reducing conditions on an 4–12% SDS-PAGE and blotting onto nitrocellulose membranes, the recombinant proteins were probed with the anti-SLe$^x$ antibody CSLEX, *right panel*, followed by an HRP conjugated secondary antibody or probed with an anti-mIgG antibody (*left panel*). *M* stands for samples from mock-transfected cells

any core enzyme, no SLe$^x$ was produced irrespective of the FucT used (not shown). On core 2, FucT-V, -VI and -VII, and to a much lesser extent, FucT-III, could all make SLe$^x$ (Fig. 1). The CSLEX and KM93 mAbs both stained SLe$^x$ on core 2 glycans of CD43/IgG and PSGL-1/IgG (Fig. 1). FucT-V, -VI, and to a low degree also -VII, contributed to SLe$^x$ biosynthesis on core 3 carried by CD43 (Fig. 2) as detected by CSLEX. However, these FucTs could not support staining by CSLEX on core 3 O-glycans on PSGL-1/IgG. The mAb KM93 did not stain SLe$^x$ on core 3 regardless of the fusion protein used (Fig. 2).

Detection of the Le$^x$ determinant with the mAb P12 revealed Le$^x$ on CD43/IgG, but not on PSGL-1/IgG, secreted by CHO cells transfected with core 3 synthase and FucT-IV, -V or -VI (Fig. 2).

Both COS and 293T cells co-transfected with FucT-III-VII, but not FucT-IV, supported SLe$^x$ biosynthesis on CD43/IgG (not shown) and PSGL-1/IgG (Fig. 3). In the latter case, SLe$^x$ expression was inconsistent following FucT-III transfection and dependent on amount of fusion protein loaded on the gel.

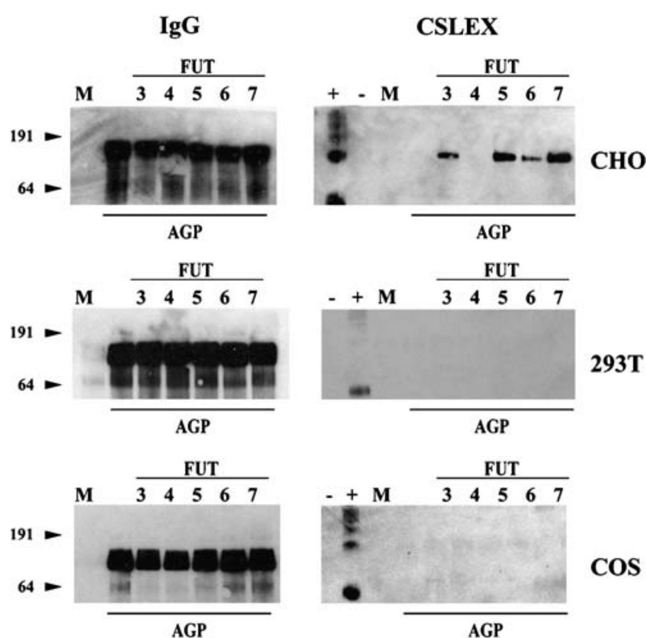


**Fig. 4** SDS-PAGE and Western blot analysis of immuno-purified AGP/mIgG$_{2b}$ proteins produced in CHO-K1, 293T and COS-7m6 cells transfected with or without different genes encoding FucTs. Recombinant proteins separated under non-reducing conditions on an 4–12% SDS-PAGE were blotted onto nitrocellulose membranes and subsequently probed with the anti-SLe$^x$ antibody CSLEX, *right panel*, followed by an HRP conjugated secondary antibody. In all lanes, similar amounts of fusion protein were loaded as shown by the anti-mIgG antibody reactivity (*left panel*). Samples from mock-transfected cells are indicated by *M*. *Positive* and *negative signs* denote BSA conjugated to SLe$^x$ and 3'sialyllactosamine, respectively

N-glycans

Only in CHO cells could we make SLe$^x$ on AGP/IgG. As with CD43/IgG and PSGL-1/IgG secreted from cells also expressing the core 2 enzyme, FucT-III, -V, -VI and -VII all supported SLe$^x$ biosynthesis on AGP/IgG (Fig. 4). No SLe$^x$ staining was found on AGP/IgG expressed together with the FucTs in 293T or COS cells (Fig. 4).

## Discussion

Our principal findings in this study are that core 2 and core 3 structures on O-glycans were fucosylated differently when carried by CD43/IgG and PSGL-1/IgG. On CD43/IgG, FucT-III, -V, -VI and -VII gave rise to SLe$^x$ on core 2 O-glycans when expressed in CHO cells, whereas only FucT-V, -VI, and to some extent also FucT-VII, could support SLe$^x$ biosynthesis on core 3, as detected with the mAb CSLEX. SLe$^x$ could also be made on core 2 O-glycans of PSGL-1/IgG following expression of FucT-III, -V, -VI and -VII. On the other hand, no SLe$^x$ at all was detected on PSGL-1/IgG carrying core 3 modifications. Of interest is also the difference in staining with the antibody KM93. It reacted well with SLe$^x$ on core 2, but did not stain the same epitope at all on core 3 O-glycans, regardless of the fusion protein used. No SLe$^x$ was detected on secreted recombinant proteins carrying O-glycans when co-expressed only with FucT-III-VII in CHO cells (data not shown). This was expected because these cells have been shown not to produce lactosamine sequences on O-glycans [31, 30, 35].

Several explanations for the difference in fucosylation of core 2 and 3 are possible. It may be that the β4GalTs in CHO cannot create type 2 extensions on core 3 carried by PSGL-1/IgG. This is unlikely, since it has been observed before [20] and it works in our system on CD43/IgG. We also know that the core 3 synthase can modify PSGL-1/IgG [20, 32]. Alternatively, either the sialyl transferases or the fucosyltransferase may not work on core 3 on PSGL-1/IgG. Also, a third possibility is that the proteins are sorted differently in the cell, resulting in PSGL-1/IgG not being modified by the enzymes needed for synthesis of SLe$^x$. It is known that transmembrane-bound and secreted forms of MUC1 from CHO transfectants have distinct glycosylation [36], but since both CD43/IgG and PSGL-1/IgG are secreted, this cannot explain our data. It is probable that core 3 on PSGL-1 is extended with galactose to form lactosamine sequences in our CHO cells [20]. Therefore, the lack of Le$^x$-reactivity (Fig. 2) of PSGL-1/IgG following FucT co-expression suggests that the FucTs do not co-localize with PSGL-1/IgG in the Golgi, supporting the third hypothesis. This is also in line with a recent report by Yano

*et al*. who suggested a sub-compartmentalization of the Golgi complex [37].

The peptide sequence of a protein can influence its O-linked glycosylation [38–43]. For mucins, the tandem repeat is important for the structure of the glycans attached [40–42, 44]. One proposed mechanism explaining the specific glycosylation of different proteins and lipids is that all glycosyltransferases have specific Golgi localizations [37, 45, 46] and that cellular trafficking of different proteins is distinct [47]. Our system provides a new approach to study the cellular mechanisms for differential glycosylation of mucins.

The fact that PSGL-1, the only protein so far known to function as a ligand for all selectins [13, 21], when modified by β3GlcNAc-T6 does not carry $SLe^x$ and also that FucT-VII poorly modified CD43 with core 3 O-glycans can explain the study by Iwai *et al*. [28]. This study showed that the core 3 synthase was down regulated in colon carcinoma, and that its expression profoundly suppressed the metastatic potential of a carcinoma cell line. It is likely that PSGL-1 and probably other proteins do not get $SLe^x$ in β3GlcNAcT-VI expressing cells, thereby reducing the number of selectin ligands which is reflected in the lowered metastatic potential [6, 48–50].

We further found that it is possible in CHO cells to express cDNAs encoding recombinant fusion proteins together with various α3FucTs to get directed expression of $SLe^x$ on N-glycans (Fig. 4). In contrast, AGP/IgG expressed in COS-7m6 and 293T cells did not stain with CSLEX (Fig. 4). The AGP/IgG secreted from COS or 293T cells did not stain with KM93 (data not shown). The absence of staining of AGP from COS and 293T might be explained by a lack of correct precursor structure on N-glycans. However, we could make $SLe^x$ on O-glycans in COS and 293T cells transfected with FucT-V-VII, as shown by the staining of purified PSGL-1 (Fig. 3).

In summary, we found that on CD43/IgG, FucT-III, -V, -VI and -VII gave rise to $SLe^x$ on core 2 O-glycans in CHO-cells, whereas only FucT-V, -VI and weakly FucT-VII were effective in producing $SLe^x$ on CD43/IgG with core 3 O-glycans. PSGL-1 did also support $SLe^x$ production by FucT-III, -V, -VI and -VII on core 2. On the other hand, no $SLe^x$ at all was detected on PSGL-1 carrying core 3 modifications. KM93 reacted well with $SLe^x$ on core 2, but did not stain this epitope at all on core 3 O-glycans, regardless of the fusion protein used. FucT-III, -V, -VI and -VII could produce $SLe^x$ on AGP in CHO cells. Thus, we show that α3FucT activity is dependent on which core saccharide and protein the sialyl-lactosamine precursor is carried. We also suggest a mechanism that can explain the lack of metastatic potential of β3GnT-VI-expressing cells [28]. Furthermore, the specificity of the widely used mAb KM93 [6, 11, 51–54] was better defined. The system based on expression of defined mucin-type proteins such as PSGL-1 and CD43 will allow us to dissect the cellular mechanisms, including sorting in the Golgi, regulating O-glycosylation.

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