α1,3Fucosyltransferase VI is expressed in HepG2 cells and codistributed with β1,4galactosyltransferase I in the Golgi apparatus and monensin-induced swollen vesicles

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The major α1,3fucosyltransferase activity in plasma, liver, and kidney is related to fucosyltransferase VI which is encoded by the *FUT6* gene. Here we demonstrate the presence of α1,3fucosyltransferase VI (α3-FucT VI) in the human HepG2 hepatoma cell line by specific activity assays, detection of transcripts, and the use of specific antibodies. First, FucT activity in HepG2 cell lysates was shown to prefer sialyl-N-acetyllactosamine as acceptor substrate indicating expression of α3-FucT VI. RT-PCR analysis further confirmed the exclusive presence of the α3-FucT VI transcripts among the five human α3-FucTs cloned to date. α3-FucT VI was colocalized with β1,4galactosyltransferase I (β4-GalT I) to the Golgi apparatus by dual confocal immunostaining. Pulse/chase analysis of metabolically labeled α3-FucT VI showed maturation of α3-FucT VI from the early 43 kDa form to the mature, endoglycosidase H-resistant form of 47 kDa which was detected after 2 h of chase. α3-FucT VI was released to the medium and accounted for 50% of overall cell-associated and released enzyme activity. Release occurred by proteolytical cleavage which produced a soluble form of 43 kDa. Monensin treatment segregated α3-FucT VI from the Golgi apparatus to swollen peripheral vesicles where it was colocalized with β4-GalT I while α2,6(N)sialyltransferase remained associated with the Golgi apparatus. Both constitutive secretion of α3-FucT VI and its monensin-induced relocation to vesicles analogous to β4-GalT I suggest a similar post-Golgi pathway of both α3-FucT VI and β4-GalT I.

Key words: fucosyltransferase/human liver/monensin/Golgi apparatus

Introduction

The α1,3fucosyltransferase (α3-FucT) enzyme activity has been detected in various types of tissues (Mollicone *et al.*, 1990, 1992; for review, see Macher *et al.*, 1991). FucTs constitute a family of homologous glycosyltransferases with a high degree of identity (Lowe, 1991). This family comprises five different fucosyltransferases, named α3-FucT III to VII (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Koszdin and Bowen, 1992; Weston *et al.*, 1992a,b; Sasaki *et al.*, 1993; Natsuka *et al.*, 1994). These enzymes differ in their capacity to transfer fucose to distinct oligosaccharide acceptors, cation requirements and tissue specific expression (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Mollicone *et al.*, 1990, 1992; Lowe, 1991; Macher *et al.*, 1991; Weston *et al.*, 1992a,b; Koszdin and Bowen, 1992, Sasaki *et al.*, 1993; Natsuka *et al.*, 1994). The five FucTs can be divided in two main subgroups, one comprising α3-FucTIV and VII, the other α3-FucTIII, V, and VI which are encoded by syntenically arranged genes. These are identical to 85% rendering their specific detection by molecular probes or immunological reagents difficult.

While the respective functions of α3-FucTIV and VII are being increasingly understood on the basis of their specific deletion in mice rendering them unable to synthesize selectin ligands (Lowe, 1997), the function of the three remaining FucTs needs to be further investigated. The study of developmental changes of tissue specific expression and the consequences of genetic polymorphisms all constitute approaches towards this goal (Mollicone *et al.*, 1994). For instance the *FUT5* and *FUT6* genes, respectively, encode two different enzymes with activities exhibiting the specificity of the plasma α1,3fucosyltransferases (Koszdin and Bowen, 1992; Weston *et al.*, 1992a). In several Java families the plasma α1,3fucosyltransferase activity has been found to be deficient. This deficiency was a result of a mutation in the *FUT6* gene (Mollicone *et al.*, 1994). The linkage relationship between a α3-FucT VI mutation and deficiency of plasma fucosyltransferase activity was further confirmed by work of van Dijk and associates (Brinkman-Van der Linden *et al.*, 1996): The missense mutation in the α3-FucT VI gene led to a complete absence of α3-fucosylation of serum glycoproteins. In sera of different individuals with inactivated *FUT6* gene but with a functional *FUT5* gene α1,3fucosyltransferase activity has not been detected (Brinkman-Van der Linden *et al.*, 1996). This finding excluded the involvement of *FUT5* in contributing to plasma FucT activity. While it seems clear that the *FUT6* gene encodes the plasma α1,3fucosyltransferase activity, its tissue origin has not formally been identified. Kidney is one candidate source of α3-FucT VI as a plasma fucosyltransferase activity. In the case of an α3-fucosylated individual with a congenital kidney anomaly, only 10% of FucT plasma activity has been detected which could be ascribed to the myeloid type (Caillard *et al.*, l988). Lack of expression of the Le^x antigen in

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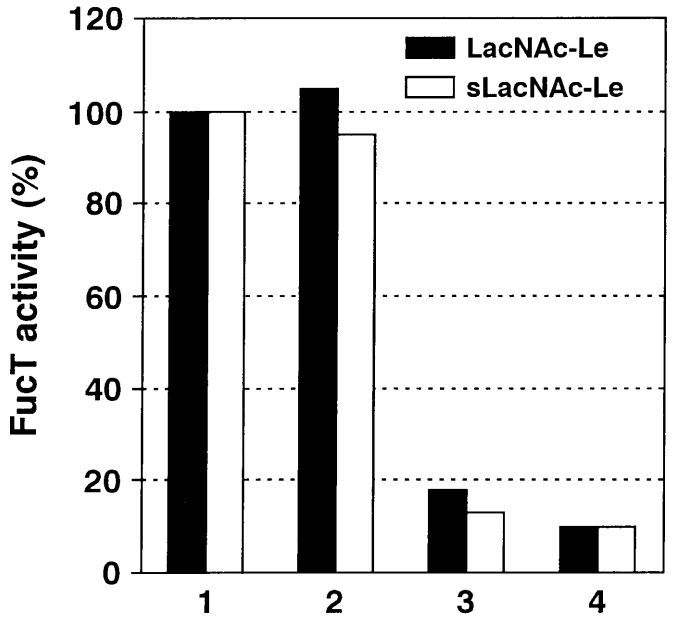

Fig. 1. Inhibition of α1,3fucosyltransferase activity in HepG2 cell lysates using the OLI antiserum. α3-FucT VI activity was assayed in lysates of HepG2 cells as described in *Materials and methods* using LacNAc and sialyl-*N*-acetyllactosamine as acceptors, respectively. 1, Control assay mixture of 50 μl supplemented with 20 μl of H_2O. 2, As in 1, with 20 μl of preimmune serum (PIS). 3, As in 1, with 10 μl of PIS and 10 μl of OLI antiserum. 4, As in 1, with 20 μl of OLI antiserum.

the kidney of this individual suggested that this organ may normally contribute to plasma FucT activity (Caillard *et al.*, l988; Mollicone *et al.*, 1990). Another candidate for the origin of a plasma FucT activity is the liver, where transcripts of α3-FucT VI and enzyme activity corresponding to α3-FucT VI have been detected (Mollicone *et al.*, 1990, 1992; Johnson *et al.*, 1995). This organ is the source of many plasma proteins. In addition, another glycosyltransferase, e.g. β1,4galactosyltransferase found in human serum originates at least partially from liver (Kim *et al.*, 1972a,b).

While α3-FucT V and α3-FucT VI were investigated as recombinant enzymes expressed in CHO cells (Borsig *et al.*, 1996, 1998) little is known on localization, intracellular transport, and release of endogenously expressed fucosyltransferases. Since the major α1,3fucosyltransferase activity in human plasma is encoded by α3-FucT VI and putatively released from the liver, we examined whether the HepG2 hepatoma cell line expresses α3-FucT VI and investigated localization, biosynthesis, intracellular transport, and release of this enzyme in these cells. In addition, β4-GalT I and α3-FucT VI were found to react similarly to monensin treatment which may indicate an analogous post-Golgi pathway (for review, see Dinter and Berger, 1998).

Results

Fucosyltransferase activity in HepG2 cells

Lack of fucosylation of liver-derived serum glycoproteins in patients affected by a deficiency of α3-FucT VI (Brinkman-Van der Linden *et al.*, 1996) prompted us to investigate α3-FucT activity in lysates of HepG2 cells, an established liver carcinoma cell line. To determine the nature of the α1,3fucosyltransferase activity detected in HepG2 cells we used the acceptor substrates listed on Table II. Acceptor substrate preference of the overall fucosyltransferase activity was directed toward type 2 acceptors, *N*-acetyllactosamine (LacNAc), and its sialylated derivative 3'-sialyllactosamine (sLacNAc). In addition, type 1 acceptor lacto-*N*-biose as well as type 6 acceptor 2'-fucosyllactose were poorly utilized (The definition of acceptor types are as follows: type 1: Galβ1→3GlcNAc; type 2: Galβ1→GlcNAc; type 6: Galβ1→4Glc). The very low ratio of utilization of type 1 to type 2 acceptors allowed to exclude expression of FucTIII to a significant amount. Preference for type 2 acceptor (neutral or sialylated) were in good agreement with the previously reported results of FucT activity in human liver cells (Jezequel-Cuer *et al.*, 1993) and in human serum (Sarnesto *et al.*, 1992). The majority of the α3-FucT activity in plasma is due to α3-FucT VI which is encoded by the *FUT6* gene (Brinkman-Van der Linden *et al.*, 1996). Indeed, a very similar acceptor specificity profile has been obtained with cloned α3-FucT VI expressed in CHO (Borsig *et al.*, 1998), COS cells (Koszdin and Bowen, 1992), or insect cells (De Vries *et al.*, 1997). To assign HepG2 cell-associated α3-FucT activity to α3-FucT VI or α3-FucT V, the enzyme activity of α1,3fucosyltransferase from HepG2 cell lysates was subjected to a neutralization experiment with a specific antiserum (designated OLI) raised against recombinant α3-FucT VI (Borsig *et al.*, 1998) (Figure 1). The OLI antiserum was able to inhibit the enzymatic activity with LacNAc and sLacNAc as respective acceptors by at least 90%. The inhibition with antibodies was similar with both acceptors suggesting the expression of either α3-FucT VI or α3-FucT V.

Table I. PCR primers for amplifying fucosyltransferases

mRNA species detected	Primers[a]
3-FucT III(585)[b]	U:5'ACCACTGGGATATCATGTCCAACCCTAAGT3'
	L: 5'GGGCCAGGTCCTTGGGGCTCTGGAAGTCG3'
α3-FucT IV(589)	U: 5'GGGGCATCCAGGCGCACACTGC3'
	L: 5'CGCTCGTAGTTGGCACGGTCTG3'
α3-FucT V(811)	U: 5'CCAGGGCTTATGGCAGTGGAACCTGTCAC3'
	L: 5'GGGCCAGGTCCTTGGGGCTCTGGAAGTCG3'
α3-FucT VI(737)	U: 5'ATCCCACTGTGTACCCTAATGG3'
	L: 5'CGGCAGGAACCTCTCGTAGTTG3'
α3-FucT VII(448)	U: 5'CCTGGGTGGTCAGCAACTTC3'
	L: 5'CGGTCACAGATGGCACAGAAAC3'

[a]U, Upper strand primer; L, lower strand primer.
[b]Numbers in parentheses indicates fragment size in base pairs.

α3-FucT VI is the only α1,3fucosyltransferase expressed in HepG2 cells

Since the OLI antiserum raised against α3-FucT VI also cross-reacts with α3-FucT V and α3-FucT III (Borsig *et al.*, 1998), further determination of the α3-FucT expressed in HepG2 cells

Table II. Measurement of Fuc-T activity in lysates of HepG2 cells and in the medium; comparison with recombinant α3-FucT VI activity from CHO cells

	Acceptor activity of α1,3fucosyltransferase				
	Relative activity[a] (%)		Activity (pmol/min/mg prot.)		
	rec. α3-FucT VI	HepG2 lysate	HepG2 medium	Lysate	Medium
N-Acetyllactosamine Galβ1→4GlcNAc	100	100	100	95	
Lacto-*N*-biose I Galβ1→3GlcNAc	0.3	<0.1	—	<0.1	nd[b]
2'-Fucosyllactose	4.5	3	—	3	nd Fucα1→2Galβ1→4Glc
3'-Sialyl-*N*-acetyllactosamine NeuAcα2→3Galβ1→4GlcNAc	131	110	117	105	2.3

[a]A value of 100% was assigned to the specific activities generated by each enzyme with the acceptor *N*-acetyllactosamine. Relative activities for each of the other acceptors were then calculated by taking the ratio of their respective specific activities to the specific activity observed with *N*-acetyllactosamine;
[b]Not determined.

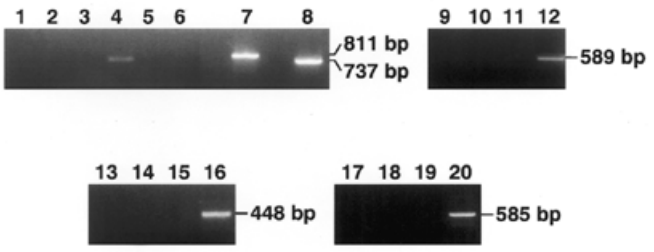

Fig. 2. RT-PCR of α1,3fucosyltransferases in HepG2 cells. After PCR analysis an aliquot was loaded on a 1.5% agarose gel containing ethidium bromide. α3-FucT V: lanes 1–3, 7; α3-FucT VI: lanes 4–6, 8; α3-FucT IV: lanes 9–12; α3-FucT VII: lane 13–16; α3-FucT III: 17–20; cDNA from HepG2 cells: lanes 1, 4, 9, 13, 17; RNA controls: lanes 2, 5, 10, 14, 18; H_2O controls: lanes 3, 6, 11, 15, 19; genomic DNA: lanes 7, 8, 12, 16, 20.

was done by RT-PCR analysis. Based on activity measurements, α3-FucT VI and α3-FucT V could be expected. To be able to distinguish between α3-FucT V and α3-FucT VI expression, we used specific primers for α3-FucT V (Borsig *et al.*, 1998) and α3-FucT VI (Table I). Absence of a signal in controls and specific amplification from cDNA and genomic DNA showed expression solely of α3-FucT VI in HepG2 cells (Figure 2, lane 4). PCR amplification with α3-FucT V primers yielded no product (Figure 2, lane 1). RT-PCR amplification with specific primers for α3-FucT III were also negative (Figure 2, lane 17). Although crossreactivity of OLI antibodies with α3-FucT IV and α3-FucT VII was not observed (data not shown), RT-PCR analysis was carried out, also with negative results (Figure 2, lanes 9, 13). Taken together, we conclude that among the five cloned α3-FucTs α3-FucT VI only is expressed in HepG2 cells.

α1,3Fucosyltransferase VI is localized to the Golgi apparatus

To determine the steady-state distribution of α1,3fucosyltransferases VI, HepG2 cells were subjected to indirect confocal immunofluorescence microscopy (Figure 3). For staining of α3-FucT VI, previously characterized polyclonal affinity purified OLI antibodies were used (Borsig *et al.*, 1998) (Figure 3C). A specific Golgi staining was found using OLI antibodies to α3-FucT VI (Figure 3C), while preimmune serum (Figure 3A) or staining with antibodies preabsorbed with rα3-FucT VI antigen (Figure 3B) produced background staining only. Golgi location of FucT was further confirmed by double confocal immunofluorescence staining with a monoclonal antibody to β1,4-galactosyltransferase I (Berger *et al.*, 1986) indicating colocalization of both antigens (Figure 3C,D).

Maturation of α3-FucT VI in HepG2 cells and its release into the medium

To determine the molecular weight of α3-FucT VI expressed in and released from HepG2 cells, immunoblotting of cell lysates and supernatants with OLI antibodies was carried out (Figure 4). In HepG2 cells, α3-FucT VI appeared as a 46.5 kDa protein, thus slightly smaller than the recombinant enzyme in CHO cells which was detected as a 47 kDa band (Borsig *et al.*, 1998). The small difference might be due to cell-type-specific glycosylation. The enzyme released from HepG2 cells migrated as a 45 kDa protein indicating an analogous processing step for the endogenously expressed enzyme as for the recombinant enzyme described previously (Borsig *et al.*, 1998; Grabenhorst *et al.*, 1998). Fucosyltransferase activity was also measured in the medium (Table II). The specificity of the released enzymatic activity was similar to the intracellular enzyme. The cumulative amount of enzyme released was half of total activity recovered in the cell lysate and in the medium. Maturation of α3-FucT VI in HepG2 cells was analyzed by metabolic labeling followed by immunoprecipitation. HepG2 cells were subjected to pulse-chase analysis (Figure 5). The 43 kDa form (no chase), corresponding to the core glycosylated enzyme, partially shifted to 46.5 kDa after 60 min, which became preponderant after 2 h. The 43 kDa form was sensitive to endo-H treatment (no chase) or PNGase treatment (not shown) and was reduced to a 36.5 kDa form indicating that all four N-glycosylation sites are occupied. After 2 h chase, endoglycosidase-H treatment reduced the mature form to ~45 kDa (chase 120 min) while converting the nonprocessed forms to 36.5 kDa. The

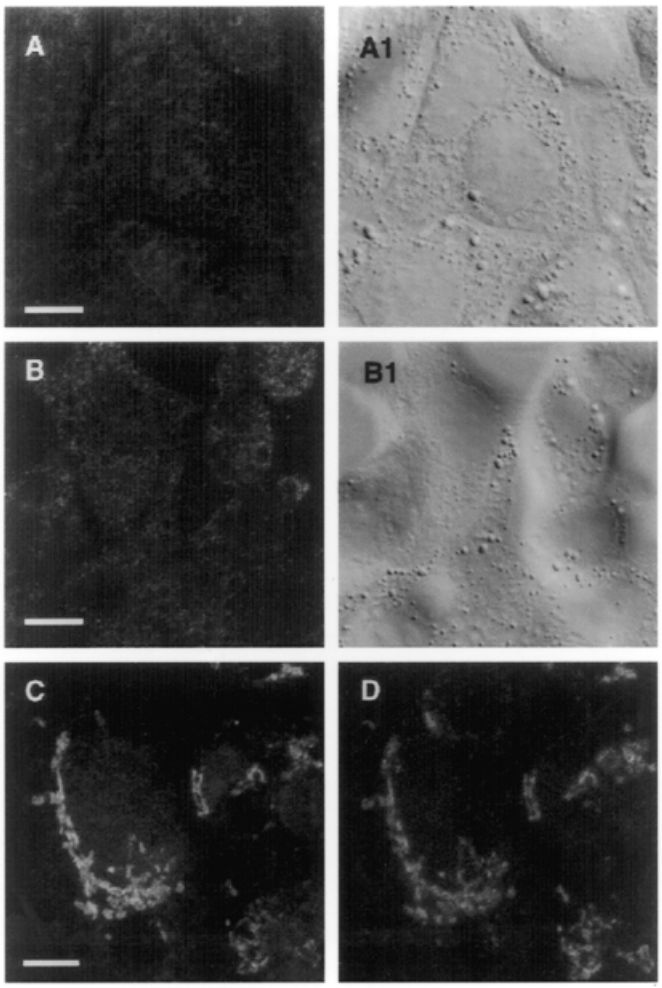

Fig. 3. Localization of α3-FucT VI in HepG2 cells by confocal immunofluorescence microscopy. Cells were grown and subjected to immunofluorescence labeling using the OLI antiserum as described in *Materials and methods*. (**A**) HepG2 cells stained with OLI preimmune serum; (**A1**) corresponding interference contrast picture (Nomarski); (**B**) HepG2 cells stained with OLI antibodies preabsorbed with srα3-FucT VI; (**B1**) corresponding interference contrast picture (Nomarski); (**C**) and (**D**) HepG2 cells double labeling for α3-FucT VI using affinity purified OLI antibodies (**C**) and β1,4-galactosyltransferase I using the mAB GT2/36/118 (**D**). Scale bar, 10 μm.

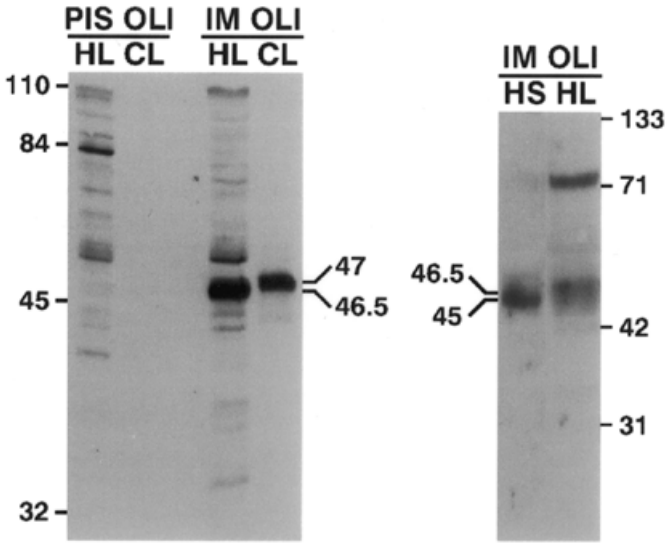

Fig. 4. Immunoblotting of α3-FucT VI in lysates and supernatants of HepG2 cells. HL, HepG2 cell lysate; CL control lysate from CHO cells stably transfected with α3-FucT VI (19); HS, HepG2 cell supernatant, see *Materials and methods*. PIS, OLI stained with OLI preimmune serum; IM, OLI stained with OLI immune serum.

partial sensitivity to endoglycosidase most likely indicates that not all of the four N-glycans are converted to complex type. In summary, maturation of α3-FucT VI expressed in HepG2 cells was almost identical to the one previously observed for rα3-FucT VI expressed in CHO cells (Borsig *et al.*, 1998).

Colocalization of α3-FucT VI with β4-GalT I in monensin-induced swollen vesicles

Previous data have shown that monensin, an established Golgi-disturbing agent (for reviews, see Mollenhauer *et al.*, 1990; Dinter and Berger, 1998), segregates β4-GalT I from sialyl-T by relocating β4-GalT I to peripheral swollen vesicles (Berger *et al.*, 1993). While the nature of these vesicles has not been unequivocally determined, they most probably belong to a post-Golgi compartment. In support of this view, β4-GalT I has been found to colocalize with TGN46 in monensin-induced swollen vesicles (Figure 6G/H). Since TGN46 is a well characterized marker of the TGN which recycles to the cell surface within the post-Golgi compartments (for review, see Banting and Ponnambalam, 1997) and which does not colocalize with β4-GalT I under steady-state conditions (Prescott *et al.*, 1997) the structures in which both TGN46 and β4-GalT I colocalize (Figure 6 G/H) are compatible with Golgi-derived vesicles. It was therefore of interest to investigate whether α3-FucT VI would conform to the same segregative behavior than β4-GalT I: HepG2 cells were treated with monensin for 30 min and analyzed by confocal immunofluorescence: As shown on Figure 6, α3-FucT VI (panel D) colocalizes in swollen vesicles with β4-GalT I (panel C) in monensin-treated cells, whereas ST6Gal I (panel B) and giantin (panel A), a putative structural protein with predominant cytoplasmic orientation (Linstedt and Hauri, 1993), remain colocalized in a nondisturbed Golgi pattern. The difference between the monensin effect on β4-GalT I and giantin is shown on Figure 6, E and F, respectively. The monensin-induced dissociation of β4-GalT I, α3-FucT VI, and TGN46 was completely reversible within 1 h after washing-out monensin (not shown). Thus, β4-GalT I and α3-FucT VI, both constitutively secreted enzymes, reacted similarly to monensin treatment but distinctly from ST6Gal I and giantin.

Discussion

In this work we present the first localization and trafficking study of an endogenously expressed fucosyltransferase. More specifically, this work deals with the presence and expression of α3-FucT VI, the product of the *FUT6* gene, in hepatocyte-derived cells. Based on α1,3fucosyltransferase activity

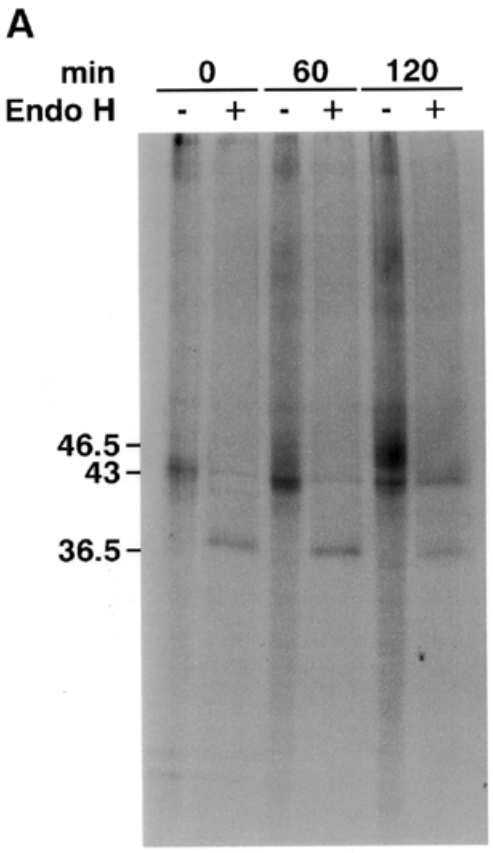

Fig. 5. Maturation of α3-FucT VI in HepG2 cells. HepG2 cells were pulsed for 20 min and chased as indicated, immunoprecipitated, and treated with endoglycosidase H as indicated. Details are described in *Materials and methods*.

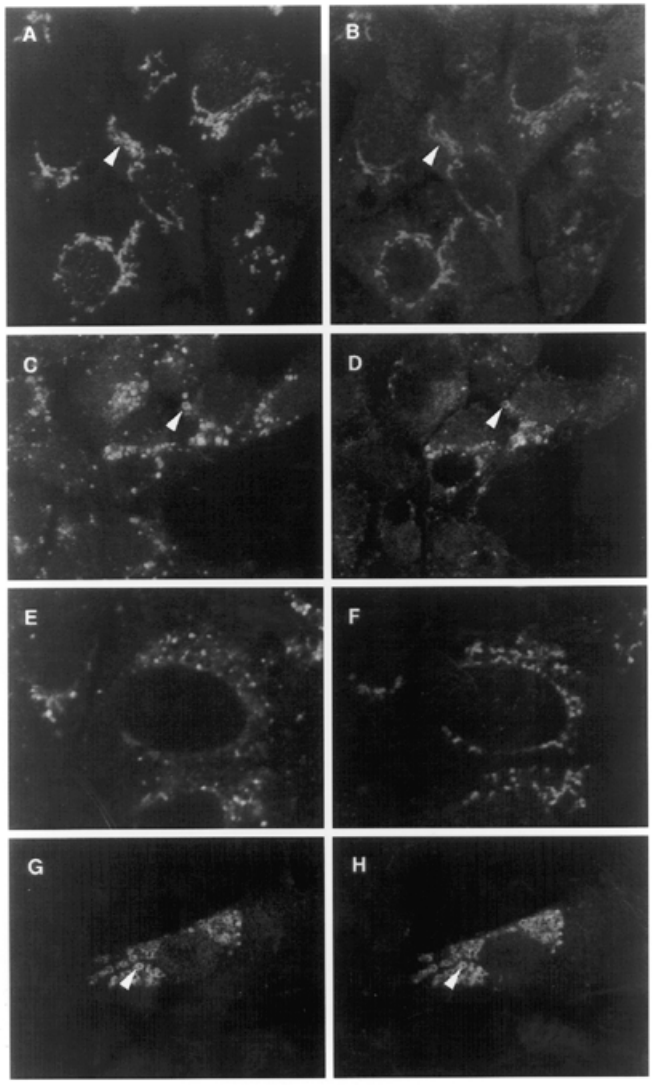

Fig. 6. Monensin selectively disturbs β4-GalT I/α3-FucT VI structural elements of the Golgi apparatus. HepG2 cells (**A**–**F**) and fibroblasts (**G**, **H**) were treated with 2 μM monensin for 30 min and processed for dual stain immunofluorescence confocal microscopy: (**A**/**B**) giantin/ST6Gal I; (**C**/**D**) β4-GalT I/α3-FucT VI; (**E**/**F**) β4-GalT I/giantin; (**G**/**H**) β4-GalT I/TGN46. Elements where colocalization is easily apparent are marked with an arrow. Scale bar, 10 μm.

measurements in human liver, α3-FucT VI was already assumed to be expressed in liver cells (Mollicone *et al.*, 1990; Johnson *et al.*, 1995). Previous work carried out by Johnson *et al*. already surmised expression of α3-FucT VI in human liver on the basis of activity measurements, immunochemical evidence using an antibody crossreactive with α3-FucT III and Northern analysis of human liver tissue and HepG2 cells. In this work we confirm and extend these findings by RT-PCR analysis of Hep-G2 cell mRNA showing the exclusive expression of α3-FucT VI among the five human α1,3fucosyltransferases cloned to date. Indeed, enzyme activity in HepG2 cells measured with different acceptors showed a very similar pattern of acceptor substrate preference as already observed by the transient expression of α3-FucT VI in COS cells (Koszdin and Bowen, 1992) and its stable expression in CHO cells (Borsig *et al.*, 1998). However, enzyme activity measurements could not unequivocally delineate the number and nature of the possible α3-FucTs which are expressed in HepG2 cells. In normal human liver, transcripts of α3-FucT V as well as α3-FucT VI have been detected (Johnson *et al.*, 1995). In the work of Mollicone and colleagues expression of at least one α1,3fucosyltransferase enzyme in liver cells was suggested (Mollicone *et al.*, 1990, 1992). To exclude expression in HepG2 cells of other members of the α3-FucT family, RT-PCR analysis was carried out. After careful adjustment of amplification conditions for each one of the cloned α3-FucTs only the expression of α3-FucT VI could be documented (Figure 2). Using antibodies specifically recognizing α3-FucT VI though crossreacting with α3-FucT III and V (Borsig *et al.*, 1998) we identified a band by immunoblotting which likely represents α3-FucT VI since the expression of both crossreactive α3-FucT III and V has been excluded on the basis of RT-PCR analysis. Moreover, α3-FucT V exceeds the size of α3-FucT VI by 15 amino acids. Thus, crossreactive α3-FucT V would migrate on SDS–PAGE differently from α3-FucT VI. α3-FucT III could be excluded on the basis of acceptor specificity: lacto-*N*-biose clearly was not a substrate (Table II) as would be expected in the case of α3 FucT III expression

(Johnson *et al.*, 1995). Here we also assign α3-FucT VI to the list of late-acting Golgi-associated glycosyltransferases. Double labeling with β4-GalT I, a *trans* Golgi enzyme (Slot and Geuze, 1983), by using confocal microscopy, suggests colocalization of both enzymes which would implicate a *trans* localization also for α3-FucT VI. Despite several efforts, ultrastructural localization of this enzyme has not been possible. Circumstantial evidence suggested the presence of recombinant α3-FucT VI in distal Golgi compartments for its ability to compete with an α2,3sialyltransferase (Grabenhorst *et al.*, 1998).

The plasma α1,3fucosyltransferase activity is encoded by the *FUT6* gene (Mollicone *et al.*, 1994; Brinkman-Van der Linden *et al.*, 1996). The origin of this activity remains unknown. The liver has been suggested to be one of the potential candidate sources for the plasma activity (Mollicone *et al.*, 1990, 1992; Johnson *et al.*, 1995). The observed release of the α3-FucT VI enzyme activity from HepG2 cells provides the first indication that the liver could be, at least in part, the source of plasma α3-FucT activity. The 50% of FucT total activity present in medium of HepG2 cells indicates efficient release from the cells. By contrast, pulse-chase analysis showed a rather slow maturation of α3-FucT VI, which reached a partial endo-H resistance only after 2 h (Figure 5). Partial endo-H resistance is a common feature for the α3-FucT VI enzyme which was already observed in stably transfected CHO cells, where even the secreted form did not reach full resistance (Borsig *et al.*, 1998). Release of α3-FucT VI occurs upon proteolytical cleavage accompanied by a reduction in molecular mass of the enzyme. Observations with other glycosyltransferases indicated that release of soluble forms of enzymes occurs by the action of serine-like (Strous and Berger, 1982; Masri *et al.*, 1988; Homa *et al.*, 1993) and cathepsin-like proteases (Weinstein *et al.*, 1987). The site of action of proteolytic processing of released glycosyltransferase remains to be determined. In this regard, it is interesting to observe that the post-Golgi fate of α3-FucT VI resembles in several aspects the fate of β4-GalT I. This enzyme is also easily detectable as a soluble glycosyltransferase in serum (Kim *et al.*, 1972a,b) and other body fluids (Gerber *et al.*, 1979), is located to the *trans* side of the Golgi apparatus (Roth and Berger, 1982; Slot and Geuze, 1983) and appears to share a common post-Golgi pathway with α3-FucT VI as inferred by their dissociation from the Golgi apparatus to Golgi-derived vesicles when cells are treated with monensin (Dinter and Berger, 1998; Berger *et al.*, unpublished observations).

The nature of the swollen vesicles induced by monensin treatment has not yet been unequivocally determined. Their appearance and location as well as codistribution of TGN46 are compatible with the view that they are TGN-derived. A number of other genuine Golgi proteins, such as ST6Gal I, giantin (as shown on Figure 6), mannosidase II, *N*-acetylgalactosaminyltransferase II and ST3Gal III (unpublished observations) also remain associated with the Golgi apparatus, indicating a specific post-Golgi behavior of β4-GalT I and α3-FucT VI.

In summary, we show that HepG2 cells harbor and secrete α3-FucT VI which is colocalized with β4-GalT I and which shows a trafficking behavior analogous to β4-GalT I.

Materials and methods

Cell culture and RNA isolation

HepG2 cells were obtained from American Type Culture Collection. They were grown in Dulbecco's modified Eagle medium (Gibco BRL) containing 10% fetal calf serum (complete medium). Total RNA from 1×10^8 HepG2 cells was isolated with guanidinium isothiocyanate followed by centrifugation on cesium chloride cushions (Sambrook *et al.*, 1989). The mRNA was isolated from the total RNA using polyT-linked Dynalbeads (Dynal, Norway) according to the manufacturer's protocol.

RT-PCR analysis of fucosyltransferases

First strand cDNA was prepared using 2 μg of poly(A)$^+$ RNA. Synthesis of cDNA was carried out with 200 U of M-MLV reverse transcriptase (Gibco BRL) and 50 pmol of oligo dT primer. For PCR of fucosyltransferases specific primers were used as depicted in Table I. For α3-FucT III 30 cycles were used as follows: 1 min 95°C, 1 min 63°C, 1 min 72°C; for FucT IV 35 cycles were used as follows: 50 s at 95°C; 40 s at 60°C; 50 s at 72°C and final extension of 5 min. For α3-FucT V and α3-FucT VI 35 cycles were used as described previously (Cameron *et al.*, 1995). For α3-FucT VII 35 cycles were used as follows: 50 s at 95°C; 40 s at 58°C; 48 s at 72°C and final extension of 5 min. PCR amplifications with 10 ng of genomic DNA for each FucT to prove the specificity of amplification were carried out. To control for genomic contaminations, each sample was amplified without reverse transcriptase or without DNA. To prove the specificity of PCR fragments, the PCR product was digested by appropriate restriction enzymes (data not shown).

Immunoblotting

HepG2 cells and CHO 61/11 cells stably transfected with recombinant α3-FucT VI were lysed in 1% (w/v) Triton X-100 in PBS. Supernatants were recovered from overnight cultures in serum-free media and concentrated 10-fold prior to analysis. Electrophoresis on 10% SDS/PAGE gel and subsequent immunoblotting was carried out as described previously (Borsig *et al.*, 1998). Nitrocellulose membranes were incubated first with affinity purified OLI antibodies (1:200) followed by goat anti-rabbit horse radish peroxidase (1:5000) and stained using the ECL developing kit according to the manufacturer's instructions (Amersham, UK).

Fucosyltransferase assay

Cell extracts containing 1% Triton X-100 were prepared as described previously (Borsig *et al.*, 1996). Protein concentrations of cell extracts were determined with a BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). A typical 50 μl reaction mixture contained 40 mM sodium cacodylate (pH 6.2), 10 mM $MnCl_2$, 10 mM L-fucose, 5 mM ATP, 101 μM GDP-fucose (~5000 c.p.m./nmol, mixture of GDP-[U-^{14}C] fucose from Amersham and GDP-fucose from Oxford Glycosciences), 5 mM of acceptor substrate (*N*-acetyllactosamine, Lacto *N*-biose I from Sigma, 3'-sialyl-*N*-acetyllactosamine or 2'-fucosyllactose from Oxford Glycosciences) and 30–60 μg of protein from cell lysates or 20–30 μl of medium. Controls without added acceptor were assayed in parallel under the same conditions. After incubation at 37°C for 2 h the reaction

mix was diluted with cold water and applied to a column containing Dowex 1X8-400, formate form (Kukowska-Latallo *et al.*, 1990). The flow-through fraction, and 2 ml of a subsequent water elution, were collected and counted with 1 volume of Instagel (Packard, IL) in a liquid scintillation counter (Rackbeta 1219, LKB). In the case of octyl-linked acceptors, assays were performed essentially as described previously (Palcic *et al.*, 1989). After stopping the assay with 1 ml of water, the assay mixture was loaded on a C18 Sep-Pak cartridge (Waters), washed three times successively with 5 ml water, and eluted with 5 ml of methanol.

Metabolic labeling and immunoprecipitation

HepG2 cells were washed with prewarmed PBS before being starved in methionine-/cysteine-free MEM medium for 20 min at 37°C. The cells were continuously labeled for 1.5 h with 50 μCi [^{35}S] methionine/cysteine (EXPRE^{35}S^{35}S methionine, cysteine labeling mix, NEN/ Du Pont, Wilmington/DE) per ml of met-/cys-free medium or 10 min (pulse/chase) with 100 μCi/ml. Cells were chased for various periods of time with complete DMEM medium and washed 2 times with ice-cold PBS. Cells were scraped off the culture dishes in 10 ml ice-cold PBS containing protease inhibitors per ml: 1 μg antipain, 1 μg aprotinin, 1 μg benzamidine, 0.5 μg leupeptin, 1 μg pepstatin A, 0.2 mM PMSF), and collected by centrifugation at 1500 × *g* for 5 min. Cells were homogenized by passing three times through a 25G$^{5}/_{8}$ gauge needle in 10 ml PBS containing 1% (w/v) Triton X-100, and lysed for 30 min at 4°C while rocking. Lysates were cleared by centrifugation for 10 min at 15,000 × *g* at 4°C and precleared for 1 h at 4°C with 100 μl suspended protein A-Sepharose (Pharmacia) in 10 ml buffer A (PBS, 1% (w/v) Triton X-100). Immunoprecipitation was carried out essentially as described before (Borsig *et al.*, 1998). Controls included preimmune serum and absorption with antigen; in both cases the specific signal was quenched (not shown). For PNG-ase F and endo-H treatment, 30 μl of 0.5% SDS, 1% β-mercaptoethanol in water was added to the washed beads and boiled for 10 min. After cooling, beads were spun down. The supernatant was adjusted to a final conc. of 1% NP-40 (w/v) and incubated for 16 h at 37°C either with 500 U of PNG-ase F (NEB, Beverly/MA) or with 50 U of endo-H (NEB). For neuraminidase treatment, to the washed beads 30 μl of 50 mM sodium citrate, pH 4.5, protease inhibitors (see above) and 50 U of neuraminidase (NEB) were added and incubated for 16 h at 37°C. The reaction was stopped by adding an equal volume of 2× SDS–PAGE sample buffer and boiled for 5 min. Immunoprecipitated proteins were separated by SDS–PAGE on a 10% acrylamide gel. After electrophoresis, gels were soaked in 50% methanol/10% acetic acid, dried, and exposed to FUJI x-ray films.

Confocal laser scanning double immunofluorescence microscopy

HepG2 cells were fixed and permeabilized as described previously (Borsig *et al.*, 1996). The first antibodies were affinity purified rabbit antibodies to α3-FucT VI (OLI) raised to soluble recombinant α3-FucT VI (Borsig *et al.*, 1998) or monoclonal antibody mAB2/36/118 to human β1,4-galactosyltransferase (Berger *et al.*, 1986), respectively. A rabbit polyclonal antiserum to TGN46, the human homologue of rat TGN38 was obtained from α3-FucT V. Ponnambalam (Dundee) Preimmune serum in an appropriate dilution was used. In case of preabsorption of OLI antibodies, affinity purified antibodies were preincubated with 10 μg of antigen for 1 h prior to the staining procedure. Fluorescein isothiocyanate (FITC) and Texas red (TR)-conjugated secondary antibodies were obtained from Dako (anti-mouse Ig) and Organon (anti rabbit Ig). For mounting of coverslips embedding medium was used as described previously (Borsig *et al.*, 1996). Immunofluorescence images were taken on a Leica microscope using dual fluorescence mode for Texas red and FITC. Single fluorescence images or extended focus projections were generated using the Imaris software (Bitplane, Zürich Switzerland).

Incubation of cells with monensin

Monolayers were incubated for the indicated times in complete medium to which a stock solution of monensin (Calbiochem) in ethanol (1 mg/ml) was added to a final concentration of 2 μM monensin (1,4 μl/ml medium); controls were supplemented with the same volume of ethanol. Recovery from monensin treatment was carried out by replacing the monensin-supplemented medium by normal medium.

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References

Banting,G. and Ponnambalam,S. (1997) TGN38 and its orthologues—roles in post-TGN vesicle formation and maintenance of TGN morphology [review]. *Biochim. Biophys. Acta Mol. Cell Res.*, **1355**, 209–217.

Berger,E.G., Aegerter,E., Mandel,T. and Hauri,H.-P. (1986) Monoclonal antibodies to soluble, human milk galactosyltransferase (lactose synthase A protein) *Carbohydr. Res.*, **149**, 23–33.

Berger,E.G., Grimm,K., Bächi,T., Bosshart,H., Kleene,R. and Watzele,M. (1993) Double immunofluorescent staining of α2,6 sialyltransferase and β1,4 galactosyltransferase in monensin-treated cells: evidence for different Golgi compartments?. *J. Cell. Biochem.*, **52**, 275–88.

Borsig,L., Katopodis,A.G., Bowen,B.R. and Berger,E.G. (1998) Trafficking and localization studies of recombinant α-1,3-fucosyltransferase vi stably expressed in cho cells. *Glycobiology*, **8**, 259–268.

Borsig,L., Kleene,R., Dinter,A. and Berger,E.G. (1996) Immunodetection of α1–3 fucosyltransferase (FucT-V). *Eur. J. Cell Biol.*,**70**, 42–53.

Brinkman-Van der Linden,E.C.M., Mollicone,R., Oriol,R., Larson,G., Van den Eijnden,D,H. and Van Dijk,W. (1996) A missense mutation in the *FUT6* gene results in total absence of α 3-fucosylation of human α (1)-acid glycoprotein. *J. Biol. Chem.*, **271**, 14492–14495 (published erratum appeared in *J. Biol. Chem.*, **271**, 267–94, 1996).

Caillard,T. Le Pendu,J., Ventura,M., Mada,M., Rault,G., Mannoni,P. and Oriol,R. (l988) Failure of expression of α-3-L-fucosyltransferase in human serum is coincident with the absence of the x (or Le^{x}) antigen in the kidney but not on leucocytes. *Immunogenetics*, **5**, 15–23.

Cameron,H.S., Szczepaniak,D. and Weston,B.W. (1995) Expression of human chromosome 19p α (1,3)-fucosyltransferase genes in normal tissues—alternative splicing, polyadenylation and isoforms. *J. Biol. Chem.*, **270**, 20112–20122.

De Vries,T., Palcic,M.P., Schoenmakers,P.S., Van Den Eijnden,D.H. and Joziasse,D.H. (1997) Acceptor specificity of GDP-Fuc:Gal β1→4GlcNAc-R α3-fucosyltransferase VI (FucT VI) expressed in insect cells as soluble, secreted enzyme. *Glycobiology*, **7**, 921–927.

Dinter,A. and Berger,E.G. (1998) Golgi-disturbing agents [review]. *Histochem. Cell Biol.*, **109**, 571–590.

Gerber,A.C., Kozdrowski,I., Wyss, SR. and Berger,E.G. (1979) The charge heterogeneity of soluble human galactosyltransferases isolated from milk, amniotic fluid and malignant ascites. *Eur. J. Biochem.*, **93**, 453–460.

Goelz,S.E., Hession,C., Goff,D., Griffiths,B., Tizard,R., Newman,B., Chirosso,G. and Lobb,R. (1990) ELFT—a gene that directs the expression of an ELAM-1 ligand. *Cell*, **63**, 1349–1356.

Grabenhorst,E., Nimtz M., Costa,J. and Conradt,H.S. (1998) In vivo specificity of human α1,3-fucosyltransferases III–VII in the biosynthesis of Lewis^x and sialyl-Lewis^x motifs on complex-type N-glycans. *J. Biol. Chem.*, **273**, 30985–30994.

Homa,F.L., Hollander T., Lehman,D.J., Thomsen D.R.and Elhammer,A.P. (1993) Isolation and expression of a cDNA clone encoding a bovine UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase. *J. Biol. Chem.*, **268**, 12609–12616.

Jezequel-Cuer,M., N'Guyen-Cong,H., Biou,D. and Durand,G. (1993) Oligosaccharide specificity of normal human hepatocyte α1–3 fucosyltransferase. *Biochim. Biophys. Acta*, **1157**, 252–258.

Johnson,P.H., Donald,A.S., Clarke,J.L. and Watkins,W.M. (1995) Purification, properties and possible gene assignment of an α1,3-fucosyltransferase expressed in human liver. *Glycoconj. J.*, **12**, 879–893.

Kim,Y.S., Perdomo,J., Whitehead,J.S. and Curtis,K.J. (1972a) Glycosyltransferases in human blood. II. Study of serum galactosyltransferase and N-acetylgalactosaminyltransferase in patients with liver disease. *J. Clin. Invest.*, **51**, 2033–2039.

Kim,Y.S., Perdomo,J. and Whitehead,J.S. (1972b) Glycosyltransferases in human blood. I. Galactosyltransferase in human serum and erythrocyte membranes. *J. Clin. Invest.*, **51**, 2024–2032.

Koszdin,K.L. and Bowen,B.R. (1992) The cloning and expression of a human α-1,3 fucosyltransferase capable of forming the E-selectin ligand. *Biochem. Biophys. Res. Commun.*, **187**, 152–157.

Kukowska-Latallo,J.F., Larsen,D.R., Nair,R.P. and Lowe,J.B. (1990) A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the lewis blood group α (1,3/1,4) fucosyltransferase. *Gene Dev.*, **4**, 1288–1304.

Linstedt,A.D. and Hauri,H.P. (1993) Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell*, **4**, 679–93.

Lowe,J.B. (1991) Molecular cloning, expression and uses of mammalian glycosyltransferases [review]. *Semin. Cell Biol.*, **2**, 289–307.

Lowe,J.B. (1997) Selectin ligands, leukocyte trafficking and fucosyltransferase genes. *Kidney Int.*, **51**, 1418–1426.

Macher,B.A., Holmes, EH., Swiedler S.J., Stults,C.L. and Srnka,C.A. (1991) Human α1–3 fucosyltransferases [review]. *Glycobiology*, **1**, 577–584.

Masri,K.A., Appert,H.E. and Fukuda,M.N. (1988) Identification of the full-length coding sequence for human galactosyltransferase (β-N-acetylglucosaminide: β1,4-galactosyltransferase). *Biochem. Biophys. Res. Commun.*, **157**, 657–663.

Mollenhauer,H.H., Morré,D.J. and Rowe,L.D. (1990) Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity [review]. *Biochim. Biophys. Acta*, **1031**, 225–246.

Mollicone,R., Gibaud,A., François,A., Ratcliffe,M. and Oriol,R. (1990) Acceptor specificity and tissue distribution of three human α-3 fucosyltransferases. *Eur. J. Biochem.*, **191**, 169–176.

Mollicone,R., Candelier,J.J., Mennesson,B., Couillin,P., Venot,A.P. and Oriol,R. (1992) Five specificity patterns of (1–3)-α-L-fucosyltransferase activity defined by use of synthetic oligosaccharide acceptors. Differential expression of the enzymes during human embryonic development and in adult tissues. *Carbohydr. Res.*, **228**, 265–276.

Mollicone,R., Reguigne,I., Fletcher,A., Aziz,A., Rustam,M., Weston,B.W., Kelly,R.J., Lowe,J.B., Oriol,R. (1994) Molecular basis for plasma α (1,3)-fucosyltransferase gene deficiency (*FUT6*). *J. Biol. Chem.*, **269**, 12662–12671.

Natsuka,S., Gersten,K.M., Zenita,K., Kannagi,R. and Lowe,J.B. (1994) Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. *J. Biol. Chem.*, **269**, 16789–16794.

Palcic,M., Heerze,L., Srivastava,O. and Hindsgaul,O. (1989) A bisubstrate analog inhibitor for α (1–2)-fucosyltransferase. *J. Biol. Chem.*, **264**, 17174–17181.

Prescott,A.R., Lucocq,J.M., James,J., Lister,J.M. and Ponnambalam,S. (1997) Distinct compartmentalization of TGN46 and β-1,4-galactosyltransferase in Hela cells. Eur. J. *Cell*, Biol., **72**, 238–246.

Roth,J. and Berger,E.G. (1982) Immunocytochemical localization of galactosyltransferase in HeLa cells: co-distribution with thiamine pyrophosphatase in *trans*-Golgi cisternae. *J. Cell Biol.*, **93**, 223–229.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sarnesto,A., Kohlin,T., Hindsgaul,O., Thurin,J. and Blaszczyk-Thurin,M. (1992) Purification of the secretor-type β-galactoside α1–2-fucosyltransferase from human serum. *J. Biol. Chem.*, **267**, 2737–2744.

Sasaki,K., Watanabe,E., Kawashima,K., Sekine,S., Dohi,T., Oshima,M., Hanai,N., Nishi,T. and Hasegawa,M. (1993) Expression cloning of a novel Gal β (1–3/1–4) GlcNAc α2,3-sialyltransferase using lectin resistance selection. *J. Biol. Chem.*, **268**, 22782–22787.

Slot,J.W. and Geuze,H.J. (1983) Immunoelectron microscopic exploration of the Golgi complex. *J. Histochem. Cytochem.*, **31**, 1049–1056.

Strous,G.J.A.M. and Berger,E.G. (1982) Biosynthesis, intracellular transport and release of the Golgi enzyme galactosyltransferase (lactose synthetase A protein) in HeLa cells. *J. Biol. Chem.*, **257**, 7623–7628.

Weinstein,J., Lee,E.U., McEntee,K., Lai, P.-H. and Paulson,J.C. (1987) Primary structure of β-galactoside α2,6-sialyltransferase. Conversion of membrane-bound enzyme to soluble forms by cleavage of the NH_2-terminal signal anchor. *J. Biol. Chem.*, **262**, 17735–17743.

Weston,B.W., Nair,R.P., Larsen,R.D. and Lowe,J.B. (1992a) Isolation of a novel human α (1,3)fucosyltransferase gene and molecular comparison to the human Lewis blood group α (1,3/1,4)fucosyltransferase gene. Syntenic, homologous, nonallelic genes encoding enzymes with distinct acceptor substrate specificities. *J. Biol. Chem.*, **267**, 4152–4160.

Weston,B.W., Smith,P.L., Kelly,R.J. and Lowe,J.B. (1992b) Molecular cloning of a fourth member of a human α (1,3)fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis x, sialyl Lewis x and difucosyl sialyl Lewis x epitopes [published erratum appeared in *J. Biol. Chem.*, 1993, 268, 18398]. *J. Biol. Chem.*, **267**, 24575–24584.