

# Trafficking and localization studies of recombinant $\alpha$ 1,3-fucosyltransferase VI stably expressed in CHO cells

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**Peripheral  $\alpha$ 1,3-fucosylation of glycans occurs by the action of either one of five different  $\alpha$ 1,3-fucosyltransferases (Fuc-Ts) cloned to date. Fuc-TVI is one of the  $\alpha$ 1,3-fucosyltransferases which is capable to synthesize selectin ligands. The major  $\alpha$ 1,3-fucosyltransferase activity in human plasma is encoded by the gene for fucosyltransferase VI, which presumably originates from liver cells. While the sequence, chromosomal localization, and kinetic properties of Fuc-TVI are known, immunocytochemical localization and trafficking studies have been impossible because of the lack of specific antibodies. Here we report on the development and characterization of a peptide-specific polyclonal antiserum monospecific to Fuc-TVI and an antiserum to purified soluble recombinant Fuc-TVI crossreactive with Fuc-TIII and Fuc-TV. Both antisera were applied for immunodetection in stably transfected CHO cells expressing the full-length form of this enzyme (CHO clone 61/11). Fuc-TVI was found to be a resident protein of the Golgi apparatus. In addition, more than 30% of cell-associated and released enzyme activity was found in the medium. Maturation and release of Fuc-TVI was analyzed in metabolically labeled CHO 61/11 cells followed by immunoprecipitation. Fuc-TVI occurred in two forms of 47 kDa and 43 kDa bands, while the secreted form was detected as a 43 kDa. These two different intracellular forms arose by posttranslational modification, as shown by pulse-chase experiments. Fuc-TVI was released to the supernatant by proteolytic cleavage as a partially endo-H resistant glycoform.**

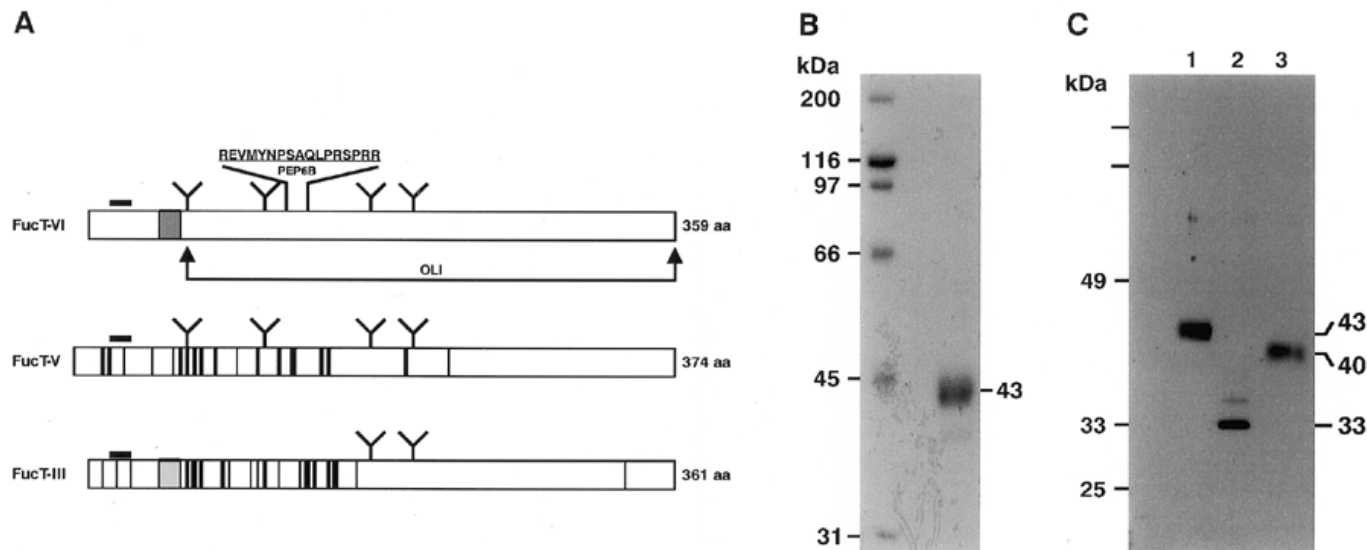
**Key words:** CHO cells/ $\alpha$ 1,3-fucosyltransferase Fuc-TVI/Golgi apparatus/secretion

## Introduction

The  $\alpha$ 1,3-fucosyltransferases (Fuc-Ts) constitute a family of glycosyltransferases with a high degree of homology (Lowe, 1991). This family of enzymes encompasses five different fucosyltransferases, named Fuc-TIII to Fuc-TVII (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Koszdin and Bowen, 1992; Weston *et al.*, 1992; Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). Fuc-Ts differ in their capacity to transfer L-fucose to oligosaccharide acceptors thereby contributing to the formation of the various Lewis antigens (Mollicone *et al.*, 1992).

While the expression of Fuc-TVII correlates with the synthesis of selectin ligands (Smith *et al.*, 1996; Maly *et al.*, 1996), the Fuc-TIV enzyme is predominantly expressed in myeloid cells with a substrate preference toward neutral lactosamine acceptors (Mollicone *et al.*, 1992; Clarke *et al.*, 1996). Three other members of this family, Fuc-TIII, Fuc-TV, and Fuc-TVI, have a very high degree of homology. They are syntenically arranged on chromosome 19 as a multigene family (Nishihara *et al.*, 1993). Their detailed acceptor specificity was recently delineated to some extent by various chimeric constructs between Fuc-TIII and Fuc-TVI (Legault *et al.*, 1995) or Fuc-TIII and Fuc-TV1995 (Xu *et al.*, 1996). These experiments showed the unexpected result that the relative preference of Fuc-TIII for type I acceptors (Lacto-*N*-biose I) can be abrogated by replacing a segment of only 50 amino acids located in the hypervariable region (AA102–150) with the corresponding segment of Fuc-TVI (Legault *et al.*, 1995). In contrast, the preference of Fuc-TIII for type I acceptors was found to be determined also by an additional region of AA73–120 as observed by domain swapping between Fuc-TIII and Fuc-TV (Xu *et al.*, 1996). Thus, the differences in acceptor substrate utilization among Fuc-TIII, -V, and -VI *in vitro* and the predominant preference for type II acceptors (*N*-acetyl-lactosamine) of Fuc-TVI and V suggests different *in vivo* functions for these enzymes. Fuc-TVI was initially described as the plasma-type enzyme, because its substrate specificity resembles that of a fucosyltransferase purified from plasma (Mollicone *et al.*, 1992; Sarnesto *et al.*, 1992). It was subsequently demonstrated that a missense mutation in Fuc-TVI gene is responsible for the deficiency in plasma activity observed in some Java families (Mollicone *et al.*, 1994). This observation demonstrates that the  $\alpha$ 1,3-fucosyltransferase activity in human plasma is indeed encoded by the Fuc-TVI gene. Moreover, on the basis of deficient fucosylation of liver glycoproteins its tissue origin has been proposed to be the hepatocytes (Brinkman-Van der Linden *et al.*, 1996).

Fucosyltransferases like other glycosyltransferases are resident membrane proteins of the endoplasmic reticulum and the Golgi apparatus. Those associated with the Golgi apparatus are type II proteins containing an *N*-terminal cytoplasmic region, a transmembrane domain, an extended stem region, and a large catalytic domain at the C-terminus. Soluble forms of some of them have been found in milk, serum, and other body fluids (for review, see Paulson and Colley, 1989). Proteolytic conversion of  $\beta$ 1,4-galactosyltransferase was observed upon release from HeLa cells (Strous and Berger, 1982). Also some cloned enzymes have been reported to be released from transfected cells (Larsen *et al.*, 1989; Jaskiewicz *et al.*, 1996; Costa *et al.*, 1997). Recently, conversion to soluble forms of three endogenously expressed glycosyltransferases was investigated in more detail. In the case of  $\beta$ 1,4-galactosyltransferase (Masri *et al.*, 1988) and O-linked *N*-acetylgalactosaminyltransferase (Homa *et al.*, 1993) the cleavage site suggested serine proteases and for  $\alpha$ 2,6-sialyltransferase the cleavage occurs by action of a cathepsin-like protease



**Fig. 1.** (A) Scheme of the primary structures of three homologous human  $\alpha$ 1-3 Fuc-Ts. Fucosyltransferases are type II membrane proteins with a short *N*-terminal cytoplasmic tail (left side), a single transmembrane domain (thick bars), a stem region containing potential *N*-glycosylation sites (stick figures) and the C-terminal luminal part involved in catalytic activity (right side). Amino acid sequence identities were determined by comparison of Fuc-TVI with Fuc-TV and Fuc-TIII. Different or missing residues are indicated with solid bars or shadowed area, respectively. The peptide (PEP6B) and soluble rFuc-TVI expressed in CHO cells (OLI) used for immunization, respectively, are indicated. (B) Coomassie staining of purified srFuc-TVI used as antigen for the induction of the OLI antiserum. rFuc-TVI was purified as described in *Materials and methods*. (C) Immunoblotting of srFuc-TVI with OLI antibodies. rFuc-TVI (1); rFuc-TVI treated with PNGase F (2); rFuc-TVI treated with endo-H (3). In each lane, 1  $\mu$ g of pure srFuc-TVI was used.

(Weinstein *et al.*, 1987). Little is known with respect to intracellular transport and trafficking of fucosyltransferases. It seems likely that Fuc-TVI as the major fucosyltransferase activity in plasma carries some distinct features with respect to release. To investigate such aspects, we first raised monospecific antibodies to recombinant Fuc-TVI and cloned stably transfected Chinese hamster ovary (CHO) cells expressing a full-length form of this enzyme. Here we report on localization, intracellular transport and release of Fuc-TVI.

## Results

### *Production and purification of soluble recombinant Fuc-TVI (srFuc-TVI)*

The D6 clone produced consistently 13–15 U/l of fucosyltransferase activity, and the pooled supernatants were kept frozen without any detectable loss of activity. Recovery at the ion exchange step was about 75%, some activity being lost in the flowthrough. To achieve maximum binding to the SP-Sepharose column it was important to lower the ionic strength of the supernatant. Recovery from the GDP-hexanolamine column was consistently almost 100% and the eluate displayed a single band by SDS/PAGE analysis (Figure 1B). Using the Bio-Rad protein assay dye, the specific activity of the final purified enzyme was found to be 14 U/mg. Amino terminal sequencing of the purified enzyme by Edman degradation gave no signal, indicating a blocked amino terminus. After digestion with trypsin, several peptides were subjected to Edman degradation and, as expected, all gave sequences corresponding to portions expected from the fucosyltransferase VI cDNA sequence (Koszdin *et al.*, 1992).

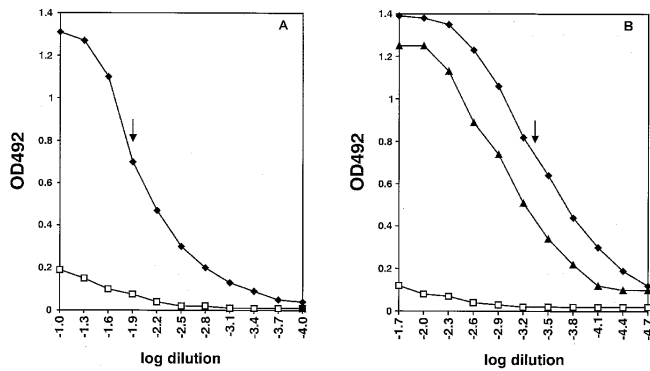
### *Characterization of antibodies*

To elicit specific polyclonal antisera to  $\alpha$ 1,3-fucosyltransferase VI (Fuc-TVI), we followed two different strategies. Because of the extensive homology of the  $\alpha$ 1,3-fucosyltransferase family (Figure 1A), we selected an amino acid stretch located in the stem region of Fuc-TVI with lowest similarity to the closely related Fuc-TV. This peptide stretch designated PEP6B was synthesized by a solid-phase method, coupled to hemocyanin, and used as an antigen. The second antiserum was produced against purified srFuc-TVI derived from CHO cells (see above). The half-maximum response to PEP6B (Figure 2A) as well as to srFuc-TVI (Figure 2B) corresponded to a titer of 1/80 and 1/2000, respectively. The antiserum to PEP6B recognized srFuc-TVI, while the antiserum to srFuc-TVI did not recognize the PEP6B peptide. The antiserum to PEP6B peptide is further designated PEP6B antiserum and the antiserum to srFuc-TVI OLI antiserum.

To ascertain the specificity of our antisera to Fuc-TVI, cell lysates from stably transfected CHO cells expressing one of the Fuc-Ts (Fuc-TVI, Fuc-TV, or Fuc-TIII) or COS cells transiently transfected with respective plasmids were subjected to immunoblotting with OLI antiserum and PEP6B antiserum, respectively (Figure 3A,B). The PEP6B antiserum was revealed to be monospecific to Fuc-TVI. Besides Fuc-TVI, the OLI antiserum also recognized Fuc-TV and Fuc-TIII. To further analyze our antisera, activity of recombinant Fuc-TVI from CHO cells was subjected to a neutralization experiment (Figure 3C). The OLI antiserum was able to inhibit activity at least by 80%, whereas PEP6B was ineffective.

### *Fuc-TVI localization in the Golgi apparatus*

To determine the steady-state distribution of Fuc-TVI expressed in stably transfected CHO cells (CHO 61/11), we subjected these cells to an indirect immunofluorescence microscopy (Figure 4) in

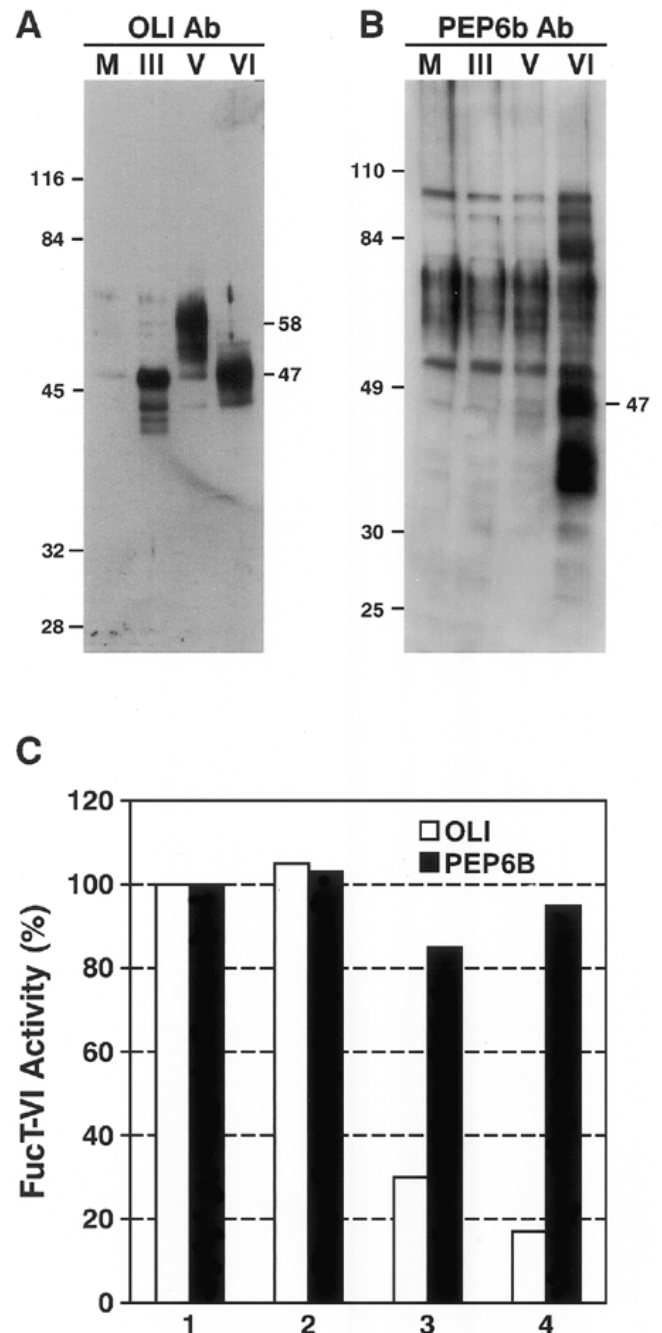


**Fig. 2.** ELISA of antisera to rFuc-TVI and to peptide PEP6B. (A) The plates were coated with peptide PEP6B. Binding curve of antiserum to PEP6B (diamonds), and PEP6B preimmune serum (squares). (B) The plates were coated with rFuc-TVI. Binding curve of OLI antiserum (diamonds), and OLI preimmune serum (squares). Binding curve of antiserum to PEP6B (triangles). Arrows indicate the titer as defined by half-maximum binding.

permeabilized cells, as described in *Materials and methods*. OLI antibodies localized Fuc-TVI to the Golgi apparatus (Figure 4E) while preimmune serum (Figure 4C) or mock transfected cells stained with antiserum (Figure 4A) showed background staining only. Golgi location of Fuc-TVI was further confirmed by double immunofluorescence staining with anti-Giantin antibodies (Linstedt *et al.*, 1993; Figure 4G,H). The Fuc-TVI staining pattern obtained with PEP6B antibodies differed considerably from that of OLI antibodies. PEP6B antibodies produced a typical ER pattern which was observed in a small number of cells only; these cells exhibited no Golgi staining (Figure 4F). These results demonstrated binding of the peptide antiserum to recombinant Fuc-TVI since neither preimmune serum (Figure 4D) nor immune serum applied to mock transfected cells produced any staining (Figure 4B). An ER like signal was also observed in some cells stained with OLI antibodies (Figure 4E) probably due to variation of expression level which may cause ER retention. Absence of Golgi staining with PEP6B antibodies indicates that the peptide epitope might become inaccessible due to post-translational modification or folding. In order to prove this possibility, CHO 61/11 were subjected to *in situ* denaturation with 6 M guanidinium-HCl prior to adding PEP6B antibodies and corresponding second antibodies (Figure 5). In fact, cells treated with guanidinium-HCl produced a strong signal in the Golgi apparatus using PEP6B antibodies (Figure 5B). Thus, recognition of Fuc-TVI by PEP6B antibodies was restricted to an unfolded form of the enzyme. Subsequent folding of the antigen prevented these antibodies from binding to their epitopes on Fuc-TVI located in the Golgi apparatus or further downstream in the secretory pathway.

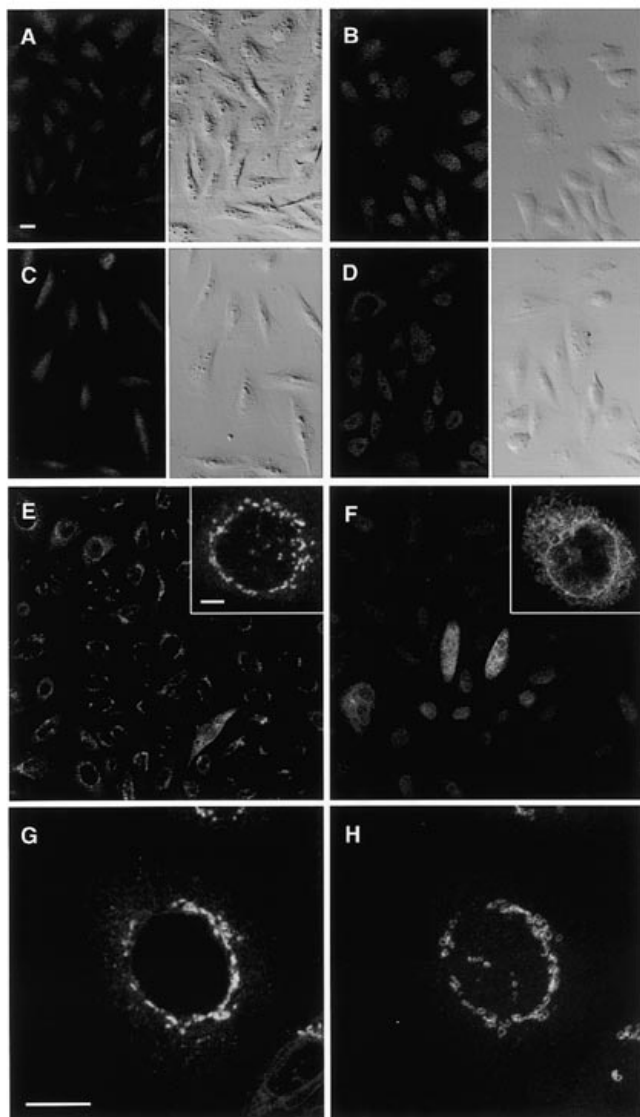
#### *Fuc-TVI in CHO 61/11 cells is secreted to the medium*

Maturation of Fuc-TVI in CHO 61/11 cells was analyzed by metabolic labeling and immunoprecipitation. Lysates of CHO 61/11 cells continuously labeled for 1 h were immunoprecipitated with OLI antibodies and analyzed on SDS/PAGE gel (Figure 6). Fuc-TVI exhibited a pattern of two major bands with molecular masses of 47 kDa and 43 kDa (lanes 2 and 3) and two minor bands of 40 kDa and 37.5 kDa, both representing putative degradation products. No shift was observed after neuraminidase treatment (lane 4). PNG-ase F treatment produced a preponderant band of



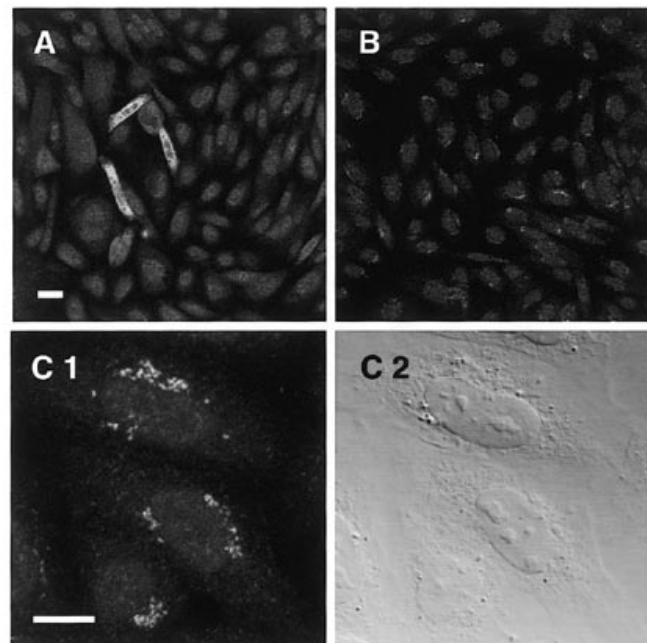
**Fig. 3.** Characterization of antibodies by immunoblotting and neutralization. Immunoblotting of cell lysates from stably transfected CHO cells (A) and transiently transfected COS cells (B) with pcDNA1 alone (M), pcFuc-TIII (III), pcFuc-TV (V), and pcFuc-TVI (VI). (A) Immunoblotting with OLI antiserum showing crossreactivity with Fuc-TIII and Fuc-TV. (B) Immunoblotting with PEP6B antiserum showing specificity only to Fuc-TVI and putative degradation products. Both immunoblots were carried out as described previously (Watzel *et al.*, 1991). (C) Inhibition of Fuc-TVI activity using OLI and PEP6B antisera, respectively. Fuc-TVI activity was assayed in lysates of CHO 61/11 cells as described in *Materials and methods* using LacNAc as an acceptor substrate. 1, Control assay mixture of 50  $\mu$ l supplemented with 20  $\mu$ l of H<sub>2</sub>O. 2, As in 1, with 20  $\mu$ l of preimmune serum (PIS). 3, As in 1, with 10  $\mu$ l of PIS and 10  $\mu$ l of the respective antiserum. 4, As in 1, with 20  $\mu$ l of the respective antiserum.

36.5 kDa and an additional band of 38.5 kDa (lane 6). Endoglycosidase-H (endo-H) treatment changed the 43 kDa to



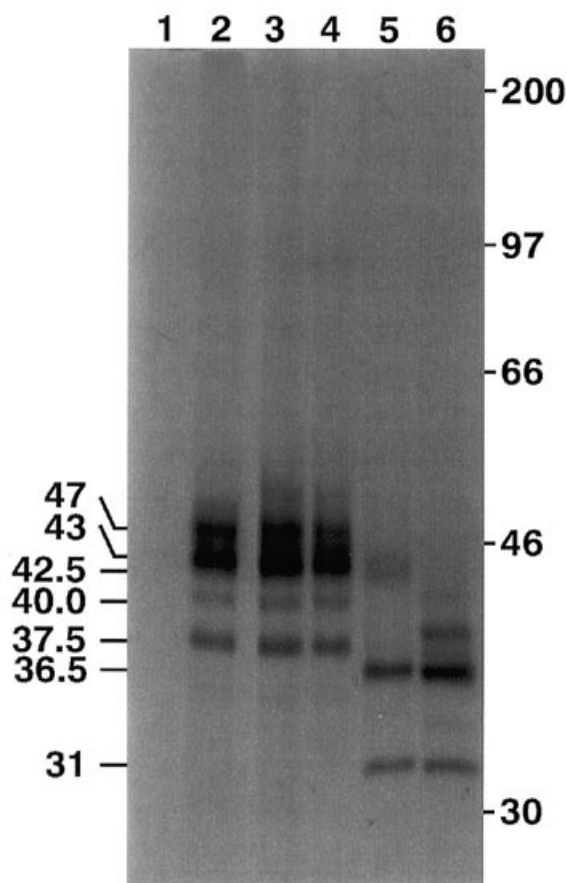
**Fig. 4.** Immunofluorescence microscopy of stably transfected CHO cells with Fuc-TVI (61/11). Cells were grown and subjected to immunofluorescence labeling as described in *Materials and methods*. (A) and (B), Control CHO cells stably transfected with pcDNAI (Mock) stained with OLI antiserum and PEP6B antiserum, respectively. (C) and (D), CHO 61/11 cells stained with OLI preimmune serum and PEP6B preimmune serum, respectively. (E) and (F), CHO 61/11 cells stained with affinity purified OLI antibodies and PEP6B antibodies, respectively. (G) and (H), Double staining of CHO 61/11 cells with OLI antibodies (G) and anti-Giantin antibodies (H). Details are given in *Material and methods*. Scale bar, 20  $\mu$ m.

36.5 kDa and the 37.5 kDa to 31 kDa forms, respectively. In addition, it gave rise to a minor form of 42.5 kDa partially sensitive to endo-H (lane 5). Fuc-TVI has four potential *N*-glycosylation sites. In case of occupancy of all four potential *N*-glycosylation sites, a shift of at least 2 kDa per site can be expected, which is in agreement with the observed shift. However, the size of 36.5 kDa form is not compatible with a full-length nonglycosylated translation product of Fuc-TVI, which is estimated to be 42 kDa. To further analyze the different



**Fig. 5.** PEP6B antibodies recognize Fuc-TVI in the Golgi apparatus of CHO 61/11 cells after *in situ* denaturation. (A) CHO 61/11 cells stained with PEP6B antibodies. (B) and (C), CHO 61/11 cells stained with PEP6B antibodies after denaturation with 6 M guanidinium-HCl (C1). Interference contrast picture of the cells shows C2. For details, see *Materials and methods*. Scale bars: (A) and (B) 20  $\mu$ m; (C) 10  $\mu$ m.

forms obtained by continuous labeling, pulse-chase labeling was carried out (Figure 7A). The 43 kDa band (no chase), corresponding to the core glycosylated enzyme, partially shifted to 47 kDa after 30 min, and became preponderant after 2 h. The 43 kDa and 37.5 kDa forms, respectively, were sensitive to endo-H treatment (no chase) and shifted to a 36.5 kDa form and a 31 kDa form, respectively, as already depicted on Figure 6 (lane 5). The 47 kDa band represented a partially endo-H sensitive band which shifted to a 42.5 kDa band (60 min chase). The shift of 4.5 kDa may correspond to cleavage of two endo-H sensitive *N*-linked glycans. After 120 min of chase the 36.5 kDa band decreased and the 42.5 kDa band became dominant. The chase medium was also subjected to immunoprecipitation (Figure 7B). Fuc-TVI was secreted into the medium after 2 h as a 43 kDa molecule. Since secretion appeared to be associated with a reduction of the molecular size by 4 kDa, we investigated this conversion further. CHO 61/11 cells were metabolically labeled and chased for 4 h. The secreted form of Fuc-TVI was immunoprecipitated and analyzed (Figure 8A). The 43 kDa band shifted upon PNG-ase F treatment to a band of 33.5 kDa (lane 4). Endo-H treatment produced a band of 41 kDa, which indicates that secreted form is also partially endo-H sensitive (lane 3). Neuraminidase treatment showed a discrete band of 43 kDa band (lane 2) in comparison to a rather diffuse band of a nontreated preparation (lane 1). The secreted and the intracellular form of Fuc-TVI were compared under reducing or nonreducing conditions (Figure 8B). The intracellular form under nonreducing condition yielded homodimers of about 94 kDa and monomers, but the secreted form appeared only as a monomer. In addition, immunoblotting also



**Fig. 6.** Metabolic labeling of CHO 61/11 cells and immunoprecipitation with OLI antibodies. Cells were grown, labeled for 1 h, and immunoprecipitated as described in *Materials and methods*. Metabolically labeled cell lysates were immunoprecipitated and glycosidase treated as follows: lane 1, OLI preimmune serum; lane 2, OLI immune serum; lane 3, affinity purified OLI antibodies; lane 4, OLI antibodies, neuraminidase; lane 5, OLI antibodies, endo-H; lane 6, OLI antibodies, PNG-ase F.

revealed a clear difference between the intracellularly matured form of 47 kDa and the secreted form of 43 kDa (Figure 8C).

**Table I.** Fuc-TVI activity in the cell lysates of CHO cells and in the medium

Acceptor	Activity of $\alpha$ 1,3-fucosyltransferase VI			
	Relative activity <sup>a</sup> (%)		Activity (nmol/min/mg prot.)	
	Lysate	Medium	Mock	Lysate
<i>N</i> -Acetylglucosamine	100	100	0	2.93
Lacto- <i>N</i> -biose I	0.3	0.2	0	0.011
2-Fucosyllactose	4.5	4.3	0	0.133
$\alpha$ 2,3-Sialyllactosamine	131	121	0	3.85
Total Fuc-TVI activity distribution	2 : 1			

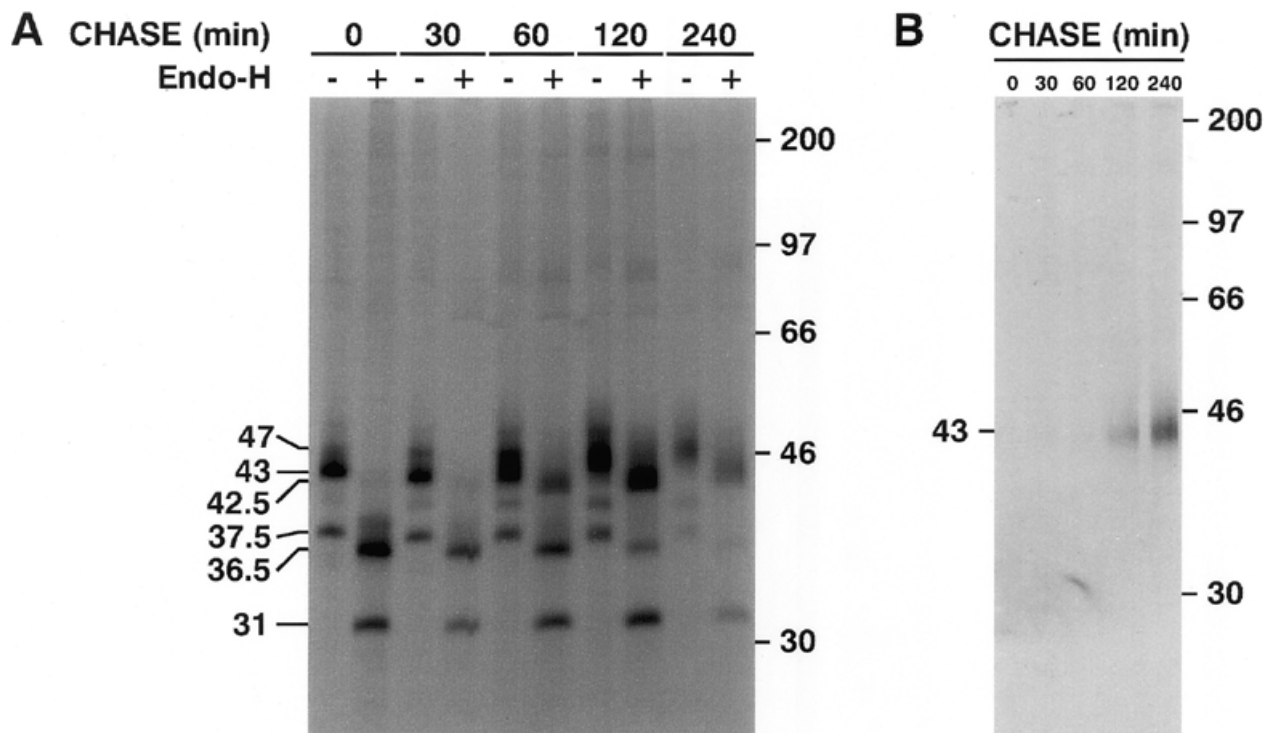
<sup>a</sup>A value of 100% was assigned to the specific activities of enzyme with the acceptor *N*-acetylglucosamine. Relative activities for each of the other acceptors are listed as the ratio of their respective activities to the activity observed with *N*-acetylglucosamine..

To define the distribution of Fuc-TVI the enzymatic activity was measured in the cell lysate and in the medium of CHO 61/11 cells after 48 h of proliferation (Table I). The activity pattern of Fuc-TVI with four different acceptors was in agreement with published results (Cameron *et al.*, 1995). No difference was observed in acceptor specificity of secreted Fuc-TVI form in comparison to the intracellular form. The enzyme activity found in the medium accounted to 30% of combined cell-associated and released enzyme.

#### Determination of Fuc-TVI molecular weight

The *N*-deglycosylated form of Fuc-TVI appeared on SDS/PAGE as a 36.5 kDa form instead of the expected 42 kDa. Therefore, we analyzed the intracellular form further. The pulse-chase experiment (see above) already indicated that early Fuc-TVI forms (no chase) had 43 kDa, which shifted to 36.5 kDa upon endo-H treatment. In these early steps after translation no proteolytic cleavage should be expected. To ascertain that Fuc-TVI is expressed as a full length enzyme without any proteolytic processing after translation, we investigated the integrity of Fuc-TVI. Thus, we prepared a cell homogenate and centrifuged it by  $100,000 \times g$  to separate the membrane fraction. An aliquot of homogenate, supernatant, and membrane pellet was analyzed on SDS/PAGE gel and immunoblotted with OLI antibodies (Figure 9). All of the immunoreactive enzyme was associated with the pellet, suggesting maintenance of membrane association. In addition, enzyme assay determinations showed that all the activity was also associated with the pellet, while in the supernatant less than 4% was detected (data not shown). Further, sodium carbonate treatment of the pellet did not extract any detectable Fuc-TVI as determined by immunoblot or by activity measurements (data not shown). This experiment showed that the Fuc-TVI present in CHO cells was membrane-associated and, therefore, can be assumed to correspond to the full length form. Moreover, in the presence of tunicamycin Fuc-TVI expressed in COS cells also migrated as a 36.5 kDa (not shown). *In vitro* translation of Fuc-TVI encoding cDNA also produced a 36.5 kDa form (data not shown). Thus, the molecular size determination of Fuc-TVI by SDS/PAGE gel seemed to underestimate its putative molecular size of 42 kDa.

To support this explanation we analyzed the glycosylation of srFuc-TVI by immunoblotting (Figure 1C). Native srFuc-TVI displayed a band of 43 kDa. Endo-H treatment generated a 40 kDa band showing partial sensitivity to endo-H even of the secreted form of Fuc-TVI and PNG-ase F treatment generated a band of 33 kDa. Since the expected molecular weight of srFuc-TVI calculated from the amino acid sequence of the cloned enzyme is 36 kDa, it is possible that srFuc-TVI migrates differently as the calibration standards on SDS/PAGE. To confirm this we analyzed the deglycosylated protein by MALDI mass spectrometry. This analysis determined the *N*-deglycosylated srFuc-TVI (after PNG-ase F treatment) to be of  $40 \pm 1$  kDa. Thus, the observed higher mobility of *N*-deglycosylated srFuc-TVI on SDS/PAGE (yielding 33 kDa) underestimates its molecular weight, which is predicted to be 36 kDa and shown to be 40 kDa by MALDI mass spectrometry. This difference of 4 kDa may be due to O-glycosylation. A similar difference in mobility is assumed to explain the discrepancy between the 36.5 kDa of the *N*-deglycosylated full length form of Fuc-TVI and the expected 42 kDa (see above).



**Fig. 7.** Pulse-chase labeling of CHO 61/11 cells. (A) Pulse was for 10 min, and chase times were as indicated. For every time point the immunoprecipitate was treated with endo-H and PNG-ase F (not shown). (B) For every time point the secreted Fuc-TVI was immunoprecipitated from medium. For details, see *Materials and methods*.

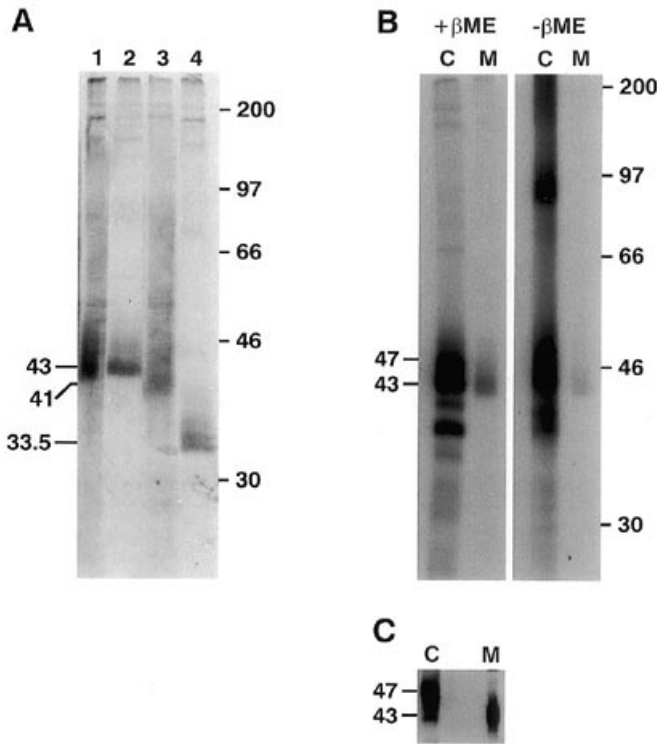
## Discussion

This work represents one of the approaches to investigate the family of  $\alpha$ 1,3-fucosyltransferases and, more specifically, aims at understanding localization and intracellular transport of Fuc-TVI. The main problem in addressing this topic is the homology of Fuc-TIII, -V, and -VI, which requires extensive antibody specificity testing to insure monospecificity. Thus, two different antibodies were raised against Fuc-TVI. As expected, OLI antibodies produced against recombinant soluble Fuc-TVI also recognized Fuc-TIII and Fuc-TV. However, PEP6B antipeptide antibody was monospecific to Fuc-TVI. The recognition of Fuc-TVI by this antibody was restricted to an unfolded protein. The PEP6B peptide stretch is closely located to the catalytic domain of the enzyme; thus, correct folding might prevent recognition by this antibody. Recent studies showed that this particular region which includes the PEP6B peptide stretch plays a critical role in substrate specificity differences between the two enzymes, Fuc-TIII and Fuc-TVI, both having almost identical C-terminal portions (Legault *et al.*, 1995). The lack of an effect of PEP6B antibodies to fucosyltransferase activity and the inability to immunoprecipitate an active enzyme form (not shown) support these observations.

By immunofluorescence microscopy using both antibodies (PEP6B and OLI) Fuc-TVI was localized to the Golgi apparatus. Similar results have been obtained with Fuc-TV in transiently transfected COS cells (Borsig *et al.*, 1996). These observations are also in agreement with the general accepted scheme for the synthesis of complex carbohydrates, which predicts that  $\alpha$ 1–3 fucosyltransferases would be localized to a late Golgi com-

partment or TGN, because of their participation in termination of glycan biosynthesis (Lowe, 1991).

The CHO 61/11 cells stably transfected with the full-length membrane-bound form of human Fuc-TVI unexpectedly secreted considerable amounts of a soluble form of the active enzyme into the medium (more than 30% of the total enzyme activity, which was measured after 48 h proliferation). Secretion of glycosyltransferases from transfected cells has been reported also for other recombinant enzymes such as Fuc-TIII expressed in BHK-21 cells (Costa *et al.*, 1997) or  $\beta$ 1,4-*N*-acetylgalactosaminyltransferase in CHO cells (Jaskiewicz *et al.*, 1996). In line with results analyzing the cleavage site of endogenously released glycosyltransferases (see *Introduction*), a common mechanism of endoproteolytic cleavage preceding secretion may exist. In the case of  $\beta$ 1,4-galactosyltransferase (Masri *et al.*, 1988) and O-linked *N*-acetylgalactosaminyltransferase (Homa *et al.*, 1993) a serine protease cleavage site was identified. Another cleavage site for a cathepsin D-like protease was identified for  $\alpha$ 2,6-sialyltransferase (Weinstein *et al.*, 1987) and for  $\beta$ 1,4-*N*-acetylgalactosaminyltransferase (Jaskiewicz *et al.*, 1996). Bulk secreted form of srFuc-TVI (Figure 1B) represents the very same form of 43 kDa as the form identified after metabolic labeling and immunoprecipitation of Fuc-TVI released from CHO 61/11 cells (Figure 8A). This observation suggests that cleavage of Fuc-TVI and its release as a soluble form occurred within the same region where srFuc-TVI was cleaved. A closer look at the sequence of the stem region and comparison with already known protease cleavage sites indicates a possible cathepsin D-like protease site. This indication needs further experimental support. The question in which compartment of the secretory pathway the soluble form of glycosyltransferase is



**Fig. 8.** Immunoprecipitation of secreted form of Fuc-TVI from medium. (A) CHO 61/11 cells were pulsed for 1.5 h and chased for 4 h. Secreted Fuc-TVI was immunoprecipitated from medium with OLI antibodies and treated as follows: lane 1, without treatment; lane 2, neuraminidase treatment; lane 3, endo-H treatment; lane 4, PNG-ase F treatment. (B) Fuc-TVI immunoprecipitates from cell lysate (C) and from medium (M) were separated on a SDS-PAGE gel under reducing condition with 5%  $\beta$ -mercaptoethanol (+ $\beta$ ME) and under nonreducing condition (- $\beta$ ME). (C) Immunoblotting of Fuc-TVI. Cell lysate (C), medium (M).

generated remains unanswered. So far this problem has only been solved in the case of  $\alpha$ 2,6-sialyltransferase showing the solubilization in the Golgi fraction (Weinstein *et al.*, 1987). In the case of Fuc-TVI a similar mechanism leading to a soluble form occurring in the Golgi apparatus can be expected. Pulse-chase labeling and immunoprecipitation clearly showed that the intracellular form of Fuc-TVI is mostly presented as homodimers and the secreted form only as monomers. This is in good agreement with a proposed model of oligomerization, which leads to retention of glycosyltransferases in the Golgi apparatus (Nilsson *et al.*, 1993). In contrast to a soluble form of  $\beta$ 1,4-*N*-acetylgalactosaminyltransferase which was secreted in the form of homodimers (Jaskiewicz *et al.*, 1996), Fuc-TVI was detected only as monomers. Homodimers of Fuc-TVI can be formed by cysteine residues present in the cytoplasmic and most probably in the transmembrane domain. A similar phenomenon of dimerization by cysteines located in the transmembrane domain followed by retention in the Golgi apparatus has been described previously for  $\beta$ 1,4-galactosyltransferase (Yamaguchi and Fukuda, 1995).

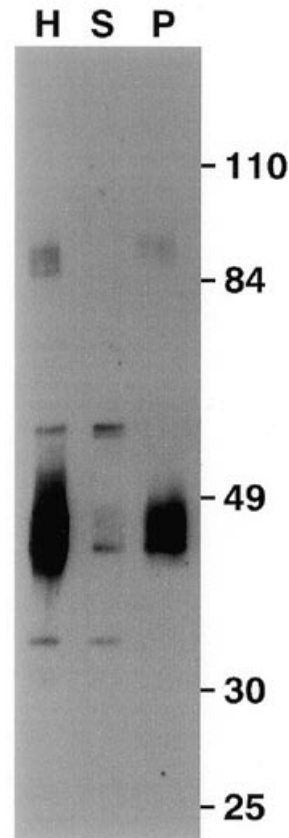
The role of released soluble glycosyltransferases remains still unanswered. The catalytic activity of these enzymes is strictly dependent on the presence of acceptors and substrates. Little is known about their access to such an environment, and whether the enzymes can be catalytically active. Soluble glycosyltransferases may have at least certain sugar binding properties. Fuc-TVI encodes an enzyme which is released to the plasma (Mollicone

*et al.*, 1992). The major part of plasma  $\alpha$ 1,3-fucosyltransferase activity is attributed to this enzyme (Brinkman-Van der Linden *et al.*, 1996). Our work is a first step to better understand the process of Fuc-TVI release into the medium. Further work on Fuc-TVI in natively expressing cells will help to understand the action of soluble and cell-surface  $\alpha$ 1,3-fucosyltransferase VI.

## Materials and methods

### Cell culture and transfection

CHO wt. cells were obtained from Dr. Renner, ETH Zürich, and were grown in HAM's medium supplemented with 10% fetal calf serum; for transfection Optimem medium was used; and for metabolic labeling MEM Select-Amin-Kit was used (all media were from Gibco BRL, Grand Island, NY). CHO cells stably transformed with pcFuc-TVI, pcFuc-TV, and pcFuc-TIII, respectively, were prepared (see below). Cells were cotransfected by lipofectamine with the respective pcDNA1 constructs and pSV2Neo (Clontech, Palo Alto, CA) in a ratio of 6:1 and on day 3 after transfection G418 was added to the medium with final concentration of 800  $\mu$ g/ml. Clones were obtained by limiting dilution and tested for fucosyltransferase activity. CHO/Fuc-TVI clone is further referred to as CHO 61/11. COS-7 cells were obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum; for transfection and metabolic labeling the same media as for CHO were used.



**Fig. 9.** Immunoblotting of Fuc-TVI in cell homogenate and a 100,000  $\times$  g pellet. CHO 61/11 cell lysate was homogenized and an aliquot was frozen and thawed twice prior to centrifugation at 100,000  $\times$  g for 1 h at 4°C. Homogenized cells (H), supernatant (S), and membrane pellet (P) were taken for SDS-PAGE and immunoblotted with OLI antibodies.

### Constructs in pcDNA1 for cell transfection

The cDNAs encoding Fuc-TV and Fuc-TVI were isolated from A-431 and HL-60 cell lines, respectively, as described previously (Koszdin *et al.*, 1992). cDNAs encoding full length forms of fucosyltransferase in pcDNA1 designated pcFuc-TVI for Fuc-TVI, pcFuc-TV for Fuc-TV, and pcFuc-TIII for Fuc-TIII were constructed as described previously (Borsig *et al.*, 1996). COS cells were transiently transfected as described previously (Borsig *et al.*, 1996).

### Peptide synthesis and coupling

Specific peptide was synthesized using standard Fmoc (*N*-[9-fluorenyl]methoxycarbonyl) chemistry on the solid phase peptide synthesizer (model 430A, Applied Biosystems, Foster City, CA). After deprotection, the synthesized peptide Pep6B (REVMYNP-SAQLPRSPRR) was purified by gel filtration on Biogel P-2 (Bio-Rad, Hercules, CA). Correct molecular weight was confirmed by fast atom bombardment mass spectrometry. For antibody production, peptide was coupled to hemocyanin using glutaraldehyde (Borsig *et al.*, 1996) and used for immunization.

### Expression and purification of recombinant soluble Fuc-TVI from CHO cells

The method of Colley and Paulson (Colley *et al.*, 1989) was used to prepare an expression construct for soluble fucosyltransferase-VI. A cassette containing the  $\gamma$ -interferon signal sequence was prepared from the following oligonucleotides: IFN10 (5'-GGA AGC TTG ACC ACC ATG AAA TAT ACA AGT TAT ATC TTG GCT TTT CAG CTC TGC-3') and IFN11 (5'-TGG GTC CTG GCA GTA ACA GCC AAG AGA ACC CAA AAC GAT GCA GAG CTG AAA AGC-3'). Oligonucleotides IFN10 and IFN11 were amplified by PCR using primers 5'-g (5'-ATA CGG AAG CTT GAC CAC CA-3') and 3'-g (5'-CTA CCC GGG GTC CTG GCA GTA A-3'). The product was digested with HindIII and SmaI restriction endonucleases and, along with a fragment of the Fuc-TVI cDNA (Koszdin *et al.*, 1992) truncated at Leu63, was cloned into pCDNA-Amp (Invitrogen). The resulting expression vector,  $\gamma$ FT-VI/pCDNA-Amp, was sequenced to confirm that the expression construct would direct the expression of the following fusion protein: MKYTSYILAFQLCIVLGS LG CYCQDP /LILL... (gamma interferon signal sequence/Fuc-TVI 63–359).

The  $\gamma$ FT-VI/pCDNA-Amp vector was transfected into COS-7 cells, and supernatants from the cells were harvested and were demonstrated to have significant fucosyltransferase activity. The 1.0 kb HindIII-BamHI fragment from  $\gamma$ FT-VI/pCDNA-Amp corresponding to the coding sequence of the fusion protein was subcloned into pREP9 (Invitrogen). This vector,  $\gamma$ FT-VI/pREP9, was electroporated into CHO cells. After selecting for resistance to G418 (0.4 mg/ml), clonal lines were isolated by limiting dilution. A particular clone, D6, gave high levels of expressed protein and was used in all subsequent work.

### Production and purification of soluble Fuc-TVI

CHO cells (clone D6) in MEM $\alpha$  (Gibco) containing 5% FBS and 0.4 mg/ml G418 were seeded in multilevel T flasks (Nunc). After the flasks became confluent the medium was changed to 3% FBS, with no added G418. Supernatants were harvested every second day and kept frozen. Pooled supernatants (about 3–5 l at a time) were concentrated on a S10Y30 spiral-wound membrane car-

tridge (Amicon) to about 10% of the original volume. The concentrate was diluted 1/3 with 20 mM Na cacodylate pH 6.5, and reconcentrated. All following steps were performed at 4°C. The final concentrate was loaded on a 50 ml column of SP-Sepharose Fast Flow (Pharmacia) equilibrated with 20 mM sodium cacodylate pH 6.5 (loading buffer) at a flow of 5 ml/min. After loading, the column was washed with 200 ml of loading buffer and subsequently with 200 ml of loading buffer containing 50 mM NaCl. The fucosyltransferase activity was eluted with 100 ml of loading buffer containing 500 mM NaCl and 10% glycerol. The eluate was concentrated to about 10% of total volume by ultrafiltration on a YM30 membrane (Amicon).

A 6 ml fucosyltransferase affinity column was prepared by attaching 40 mg of GDP-hexanolamine to 3 g activated CH Sepharose 4B (Pharmacia) according to the manufacturer's instructions and finally equilibrated with 20 mM sodium cacodylate pH 6.5, 10% glycerol, 20 mM Mn<sup>2+</sup> (affinity buffer). After ion exchange chromatography, the concentrated fucosyltransferase eluate was diluted with an equal volume of affinity buffer and loaded on to the GDP-hexanolamine column at a flow rate of 0.5 ml/min. The column was subsequently washed with 100 ml of affinity buffer containing 500 mM NaCl in order to remove nonspecifically bound proteins and then with 20 ml 20 mM sodium cacodylate pH 6.5, 10% glycerol, 500 mM NaCl. The fucosyltransferase activity was eluted with 50 ml 20 mM sodium cacodylate pH 6.5, 20% glycerol, 500 mM NaCl, 10 mM EDTA, 10 mM GDP. The final eluate was concentrated to a final volume of a few ml, exchanged two times against 20 ml of 20 mM sodium cacodylate, 20% glycerol, 200 mM NaCl, 0.02% Na azide and stored at 4°C.

### Antibody production and purification

Polyclonal sera against peptide antigen Pep6B, and srFuc-TVI were raised as described previously (Borsig *et al.*, 1996). The antibodies were affinity-purified by passing over columns of immobilized peptide on Affi-Gel 10 support (Bio-Rad) and eluted as described previously (Borsig *et al.*, 1996).

### ELISA

Nunc immunoplates Maxisorp (Gibco) were coated with 0.5  $\mu$ g/ml of peptide or 1  $\mu$ g/ml of srFuc-TVI and used as described previously (Watzel *et al.*, 1991).

### Confocal laser scanning double immunofluorescence microscopy

CHO cells and COS-7 cells were fixed and permeabilized as described previously (Borsig *et al.*, 1996). As first antibodies PEP6B antibodies, OLI antibodies, or anti-Giantin mAB G1/133, kindly provided by Prof. H. P. Hauri, Biocenter Basel (Linstedt *et al.*, 1993), were used. Fluorescein isothiocyanate (FITC) and Texas red (TR)-conjugated secondary antibodies were obtained from Dako (anti-mouse Ig) and Organon (anti rabbit Ig). Embedding medium containing 5% n-propyl-gallate in 30 mM Tris-HCl, pH 9.5 with 70% of glycerol for mounting of coverslips was used. In case of *in situ* denaturation of CHO 61/11 cells, 6 M guanidinium HCl was added on the coverslips prior to adding of antibodies. After 30 min treatment cells were washed six times for 5 min with PBS, and then first and second antibodies were used. 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).



### Metabolic labeling

CHO cells were plated on 100-mm Petri dishes and grown till 80% confluency. Before labeling, cells were washed with PBS and incubated for 15 min at 37°C in methionine, cysteine-free MEM medium, and then labeled for 1 h at 37°C (continuous labeling) or 10 min (pulse/chase) with 0.4 mCi/plate [<sup>35</sup>S] methionine (EXPRE<sup>35</sup>S<sup>35</sup>S methionine, cysteine labeling mix, NEN/Du Pont, Wilmington, DE). Cells were washed or chased with complete DMEM medium and two times with PBS. In case of CHO 61/11 cells labeling for secreted form of Fuc-TVI, cells were labeled for 1.5 h and chased in complete medium for 4 h. 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 benzamide, 0.5 µg leupeptin, 1 µg pepstatin A, 0.2 mM PMSF), and collected by centrifugation at 1500 × g for 5 min.

### Immunoprecipitation and electrophoresis

Cells were lysed in 1 ml PBS containing 1% (w/v) Triton X-100 for 30 min at 4°C while rocking. Lysates were cleared by centrifugation for 10 min at 15,000 × g at 4°C and preabsorbed for 1 h at 4°C with 100 ml suspended protein A–Sepharose (Pharmacia) in 10 ml buffer A (PBS, 1% (w/v) Triton X-100). Protein-A beads were spun down and antiserum or purified antibodies bound to 100 ml suspended protein A–Sepharose were added to the precleared lysate, respectively. Immunoprecipitation was carried out overnight by 4°C while rocking. The Sepharose beads were washed three times with buffer A and three times with PBS, after which they were boiled for 5 min in reducing SDS–PAGE sample buffer. 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 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 and dried, and dried gels were exposed to FUJI X-RAY films.

### Immunoblotting

Cell lysates were prepared in 1% (w/v) Triton X-100 in PBS. Electrophoresis on 10% SDS/PAGE gel and following immunoblotting was carried out as described previously (Watzel *et al.*, 1991). Nitrocellulose membranes were incubated first with affinity purified PEP6B antibodies or OLI antibodies (1:200) followed by goat anti rabbit-horseradish peroxidase (1:5000) and stained with ECL developing kit according to the manufacturer's instructions (Amersham).

### Fucosyltransferase assay

Cell extracts containing 1% Triton X-100 were prepared from CHO cells as described previously (Kukowska-Latallo *et al.*, 1990). 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<sub>2</sub>, 10 mM L-fucose, 5 mM ATP, 101 µM GDP-fucose (~5000 c.p.m./nmol, mixture of GDP-[U-<sup>14</sup>C] fucose from Amersham and GDP-fucose from Oxford Glycosciences), 5 mM of acceptor substrate (*N*-acetylglucosamine, lacto *N*-biose I from Sigma, α<sub>2</sub>,3-sialyllactosamine 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 srFuc-TVI, assays were performed essentially as described by Palcic *et al.*, using LacNAc-octyl as an acceptor and assay conditions as described above (Palcic *et al.*, 1988). 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.

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### Abbreviations

BSA, bovine serum albumin; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; endo-H, endoglycosidase H; FBS, fetal bovine serum; Fuc-T, α<sub>1</sub>,3-fucosyltransferase; srFuc-T, soluble recombinant Fuc-T; GDP-fucose, guanosine diphospho-l-fucose; LacNAc-octyl, Galβ<sub>1</sub>,4GlcNAcβO-(CH<sub>2</sub>)<sub>8</sub>-CO<sub>2</sub>-Me; MALDI, matrix-assisted laser desorption ionization; PNG-ase F, peptide-*N*-glycanase F.

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