Glycoconjugate Journal 17, 859–865, 2000

Glycosylation of the N-terminal potential N-glycosylation sites in the human α1,3-fucosyltransferase V and -VI (hFucTV and -VI)

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Human α1,3-fucosyltransferase V and -VI (hFucTV and -VI) each contain four potential N-glycosylation sites (hFucTV: Asn60, Asn105, Asn167 and Asn198 and hFucTVI: Asn46, Asn91, Asn153 and Asn184). Glycosylation of the two N-terminal potential N-glycosylation sites (hFucTV: Asn60, Asn105 and hFucTVI: Asn46 and Asn91) have never been studied in detail. In the present study, we have analysed the glycosylation of these potential N-glycosylation sites. Initially, we compared the molecular mass of hFucTV and -VI expressed in COS-7 cells treated with tunicamycin with the mass of the proteins in untreated cells. The difference in molecular mass between the proteins in treated and untreated cells corresponded to the presence of at least three N-linked glycans. We then made a series of mutants, in which the asparagine residues in the N-terminal potential N-glycosylation sites were replaced by glutamine. Western blotting analyses demonstrated that both sites in hFucTV were glycosylated, whereas in hFucTVI only one of the sites (Asn91) was glycosylated. All the single mutants and the hFucTVI N46Q/N91Q double mutant exhibited enzyme activities that did not differ considerably from the wt activities. However, the enzyme activity of the hFucTV N60Q/N105Q double mutant was reduced to approximately 40% of the wt activity. In addition, castanospermine treatment diminished the enzyme activity and hence trimming of the N-linked glycans are required for expression of full enzyme activity of both hFucTV and -VI. The present study demonstrates that both of the N-terminal potential N-glycosylation sites in hFucTV and one of the sites in hFucTVI are glycosylated. Individually, their glycosylation does not contribute considerably to expression of enzyme activity. However, elimination of both sites in hFucTV reduces the enzyme activity.

Keywords: **fucosyltransferases, glycoprotein, N-glycosylation, site-directed mutagenesis**

Abbreviations: **CS, castanospermine; FucTs, α1,3/4-fucosyltransferases; hFucTIII, α1,3/4-fucosyltransferase III; hFucTV, α1,3-fucosyltransferase V; hFucTVI, α1,3-fucosyltransferase VI; hFucTs, human α1,3/4–fucosyltransferases; HRP, horseradish peroxidase; PBS, phosphate buffered saline; TM, tunicamycin; TMD, transmembrane domain; wt, wild type.**

Introduction

The α1,3/4-fucosyltransferases (FucTs) catalyse the transfer of fucose from GDP-fucose to sub-terminal and/or internal *N*-acetylglucosamine residues on glycoproteins and glycolipids. At present six human FucTs (hFucTs) genes have been cloned [1–9]. They all share a similar general structure, which consists of a short N-terminal cytoplasmic domain, a transmembrane domain, and a luminal domain containing a stem region and a globular catalytic domain. All the hFucTs contain potential N-glycosylation sites in the catalytic domain.

The human α1,3-fucosyltransferase V (hFucTV) contains four potential N-glycosylation sites at Asn60, Asn105, Asn167 and Asn198. The human α1,3-fucosyltransferase VI (hFucTVI) also contains four potential N-glycosylation sites at Asn46, Asn91, Asn153 and Asn184 that are homologous to the four sites in hFucTV. Two of the N-glycosylation sites in hFucTV and -VI are situated in the N-terminal part of the two enzymes and the other two are situated closer to the C-terminal part (see Figure 1A). The human α1,3/4-fucosyltransferase III (hFucTIII), which displays a high overall amino acid sequence similarity with hFucTV and -VI contains only two potential N-glycosylation sites one at Asn154 and the other at Asn185.

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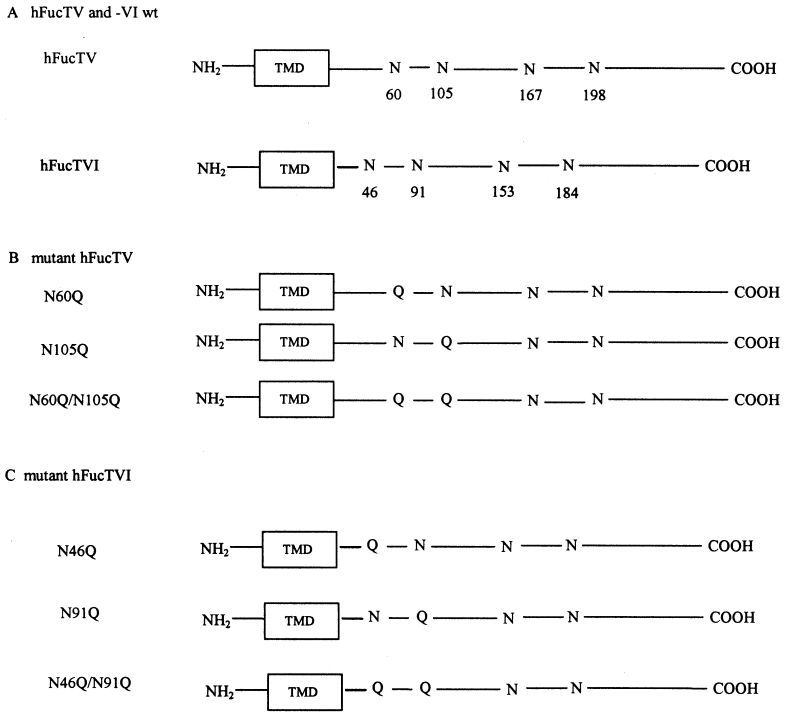

Figure 1. Structure of the hFucTV and hFucTVI wt's and mutants. The four potential N-glycosylation sites in hFucTV and -VI are all situated in the catalytic domain (A). Mutant hFucTV and -VI generated in this study by site-directed mutagenesis: hFucTV (B) and hFucTVI (C). TMD, transmembrane domain.

The two potential N-glycosylation sites of hFucTIII are homologous to the two C-terminal sites of hFucTV and -VI. The role of the potential N-glycosylation sites in the hFucTs and in FucTs from other species has only been analysed in a few studies. Recently, we demonstrated that the two conserved C-terminal N-glycosylation sites in hFucTIII, -V and -VI are glycosylated when the proteins are expressed in COS-7 cells and that their glycosylation is required for the expression of full enzyme activity of all three enzymes. Furthermore, we showed that lack of glycosylation at the Asn185 of hFucTIII did not change the K_m values for the oligosaccharide acceptor and the nucleotide sugar donor [10]. In addition, a recent study on rat α1,3-fucosyltransferase IV (rFucTIV) expressed in COS-1 cells demonstrated that N-glycosylation of both of its potential N-glycosylation sites is necessary for expression of full enzyme activity. However, lack of glycosylation at any of the sites or at both sites did not affect the intracellular location of the enzyme [11]. Furthermore, it has been demonstrated, using domain swapping and site-directed mutagenesis that Asn105 of hFucTV, and Asn46 and Asn91 of hFucTVI can be eliminated without any loss of catalytic activity [12,13]. In addition, there was not indication that the Asn105 of hFucTV was glycosylated [12]. The actual glycosylation state of Asn60 of hFucTV, and Asn46 and Asn91 of hFucTVI has not been analysed. Analyses of the role of potential N-glycosylation sites in other glycosyltransferases have demonstrated that their roles vary from protein to protein. As for other glycoproteins, N-linked glycans are required for full enzyme activity of some glycosyltransferases, whereas in others elimination of the N-glycosylation sites had no apparent effect on enzyme activity [14–19]. In addition, the N-linked glycans of glycosyltransferases are involved in protein folding, prevention of proteolytic degradation and intracellular transport and localisation [15–18,20].

The present study investigates the glycosylation of the two N-terminal potential N-glycosylation sites of hFucTV and -VI using glycosylation and processing inhibitors, and site directed mutagenesis. The obtained results show that both Asn60 and Asn105 of hFucTV are glycosylated when expressed in COS-7 cells, whereas Asn91 but not Asn46 is glycosylated in hFucTVI. Individually, none of the four N-glycosylation sites contribute considerably to the expression of α1,3-fucosyltransferase activity. However, elimination of both sites in hFucTV reduced the enzyme activity to approximately 40% of the hFucTV wt activity. In addition, we demonstrate that trimming of the N-linked glycans of both hFucTV and -VI is indispensable for the expression of full enzyme activity.

Materials and methods

Materials

Unique Site Elimination (U.S.E) Mutagenesis Kit, ECL+ Plus Western Blotting Detecting System, ECL protein molecular mass markers and GDP-[U-^{14}C]fucose were purchased from Amersham Pharmacia Biotech, Uppsala,

Sweden. The mammalian expression vector pCDNA3.1 was from Invitrogen, San Diego, CA, USA. BigDye™ terminator cycle sequencing kit with AmpliTaq polymerase FS was purchased from Applied Biosystems, Foster City, CA, USA. The *N*-acetyllactosamine (Galβ1-4GlcNAc) was obtained from Dextra Laboratories, Reading, United Kingdom. Tunicamycin, castanospermine and FUGENE™ 6 Transfection Reagent were from Roche Molecular Biochemicals, Basel, Switzerland. The anion exchange resin (AG 4-X4) was delivered from Bio-Rad. NuPAGE gels (10%) were purchased from NOVEX. The PVDF plus transfer membrane was from Micron Separations Inc., Westborough, MA, USA. Horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG was from DAKO, Copenhagen, Denmark. The rabbit polyclonal antiserum to recombinant hFucTVI (OLI-antiserum) was kindly provided by Professor Eric G. Berger, Institute of Physiology, University of Zurich, Switzerland. The selection primers and the target mutagenic primers were synthesised by Hobolt DNA Syntese, Hillerod, Denmark.

Site-directed mutagenesis and cloning of the *hFUT5* and *hFUT6* wt's and mutants

Cloning of the *hFUT5* and *hFUT6* wt genes has been described previously [10]. Site directed mutagenesis was performed using U.S.E Mutagenesis Kit. The kit is based on the unique site elimination procedure developed by Deng and Nickoloff [21], which uses a two-primer system to generate site-specific mutations. The selection primer eliminates a unique non-essential restriction site, which subsequently serves as selection of mutated plasmids. The target mutagenic primer(s) introduce(s) the desired mutation(s) in the gene of interest. The target mutagenic primers used to replace the Asn residues by Gln in the two N-terminal potential glycosylation sites of hFucTV and -VI were as follows: 5′-CGGGGCTCCCCAGGGGTCCCGCTG-3′ for N60Q, 5′CGGCCGACTGCCAGATCACTGCCG-3′ for N105Q, 5′-CTGTGTACCCTCAGGGGTCCCGC-3′ for N46Q and 5′-GGCTGACTGCCAGATCACTGCCG-3′ for N91Q. The selection primers used to eliminate a unique non-essential restriction site (*Mfe1 or Xho 1*) in the pCDNA3.1 vector were as follows: 5′GCAAGGCTTG-ACCGACGCGCGCATGAAGAATCTGC-3′ for *Mfe1* and 5′ CAGTGGCGGCCGCTGGAGTCTAGAGGGCCC-3′ for *XhoI* (used only for the generation of the hFucTV N60Q/N105Q double mutant). The mutations introduced by the primers are underlined. The target mutagenic primers and the selection primers were phosphorylated at the 5′ end and purified by HPLC. Following the mutagenesis reactions the different *hFUT5* and *hFUT6* mutants were sequenced in both directions to verify the presence of the introduced mutations and to exclude the presence of other mutations. Sequencing was performed using an ABI PRISM 310 DNA sequencer and the BigDye™ terminator cycle sequencing kit with AmpliTaq polymerase FS.

Transient expression of hFucTV and -VI wt's and mutants in COS-7 cells and tunicamycin and castanospermine treatments

Transfection and subsequent expression of wt hFucTV and -VI and the mutants were performed using FUGENE™ 6 Transfection Reagent and were carried out as described previously [10]. The tunicamycin or castanospermine treated COS-7 cells were also transfected using FUGENE™ 6 Transfection Reagent. Tunicamycin (1 μg/ml) or castanospermine (50 μg/ml) were added to the cells 6 h after transfection. After additional 16 h the cells were harvested.

Measurement of α1,3-fucosyltransferase activity

The α1,3-fucosyltransferase activity of wt hFucTV and -VI and the mutants in transfected COS-7 cells was measured using a modification of a previously published procedure [22]. COS-7 cells were lysed in phosphate-buffered saline (PBS) containing 1% Triton X-100. Twenty μl of the cell lysates were added to 80 μl of a reaction mixture containing 25 mM Tris HCl (pH 7.2), 0.5% Triton X-100, 10 mM $MnCl_2$, 5 mM ATP, 2.5 μM GDP-[U-^{14}C]fucose (~150 000 cpm) and 5 mM *N*-acetyllactosamine (oligosaccharide acceptor). The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped with 1 ml of cold water and the entire solution was applied onto a 2-ml column packed with anion exchange resin (AG 4-X4). The column was washed with 5 ml of water and incorporation of [^{14}C]fucose was determined by liquid scintillation counting of the flow-through fraction. Cell lysate volume was used to calculate the enzyme activity since this was the most reproducible normalizer for enzyme activity.

SDS-PAGE and Western blotting analyses

Cell lysates (approximately 2 μg of protein) were heated to 70 °C in SDS-sample buffer with 5% mercaptoethanol and electrophoresed on a 10% NuPAGE gel. Subsequently, the separated proteins were electrophoretically transferred to a PVDF-plus membrane at 200 mA for 1 h. Non-specific binding sites on the PVDF membrane were blocked with 2% BSA and 0.1% tween in PBS. The hFucTV and -VI proteins were detected using the OLI antiserum (1 : 300 dilution) followed by HRP-conjugated swine anti-rabbit IgG (1 : 10000 dilution). The OLI antiserum is a rabbit polyclonal antiserum against recombinant hFucTVI, which crossreacts with hFucTIII and -V as described by Borsig *et al.*, 1998 [23]. The immunoreactive bands were visualized with ECL + Plus Western Blotting Detection System.

Results and discussion

Effect of tunicamycin and castanospermine on hFucTV and hFucTVI expressed in COS-7 cells

COS-7 cells transfected with wt hFucTV or -VI were treated with tunicamycin (TM) to compare the apparent molecular mass of hFucTV and -VI in tunicamycin treated cells with the

molecular mass of the proteins in untreated cells. In addition, the role of glucose trimming of the N-glycans was studied by treating the hFucTV or -VI transfected COS-7 cells with castanospermine (CS). Western blotting analysis was performed using OLI antiserum (Figure 2). The wt hFucTV and -VI migrated with a molecular mass of ~48 kDa and ~44 kDa respectively (Figure 2, lane 1 and lane 4). The hFucTV and -VI protein bands from tunicamycin treated cells migrated faster (hFucTV ~37 kDa and hFucTVI ~35 kDa) than in untreated cells (Figure 2, lane 3 and lane 6) due to the lack of N-linked glycans. The difference in molecular mass (~9–11 kDa) between the enzymes in untreated and TM treated COS-7 cells corresponds to the mass of at least three oligosaccharide chains. These results indicate that in addition to the C-terminal glycosylation sites, one or both of the N-terminal potential N-glycosylation sites of hFucTV and -VI are indeed glycosylated. The hFucTV and -VI from CS treated cells migrated a little above (hFucTV ~52 kDa and hFucTVI ~47 kDa) the enzymes from untreated cells (Figure 2, lane 2 and lane 5) due to the inhibition of glucose trimming. The steady state levels of hFucTV and -VI in treated and untreated cells are comparable.

There is a discrepancy between the observed molecular mass of wt hFucTV and -VI in untreated cells (hFucTV ~48 kDa and hFucTVI ~44 kDa) and the one predicted from their amino acid sequence plus 3–4 oligosaccharide chains of approximately 2–3 kDa each (hFucTV ~49–55 kDa and hFucTVI ~47–53 kDa). This was also observed when hFucTIII was detected by Western blotting [2,10,23]. The upper bands at around 97–68 kDa (Figure 2, lanes 1–6) most probably are hFucTV and -VI dimers. Apart from their molecular mass, this is supported by the observation that the migration of these bands in the lysates of inhibitor treated COS-7 cells (Figure 2, lanes 2, 3, 5 and 6) changes in a similar way to the lower hFucTV and -VI bands.

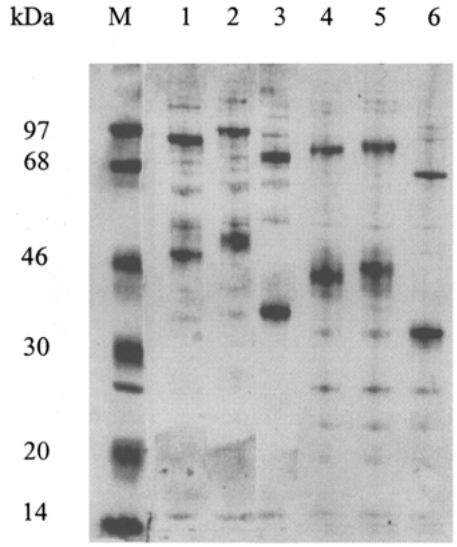

Figure 2. Western blotting analysis of wt hFucTV and -VI in transfected COS-7 cells with or without addition of tunicamycin (TM) or castanospermine (CS). Lane M, ECL protein molecular mass markers; lane 1, hFucTV wt in COS-7 cells in the absence of inhibitor; lane 2, hFucTV wt in COS-7 cells treated with CS; lane 3, hFucTV wt in COS-7 cells treated with TM; lane 4, hFucTVI wt in COS-7 cells in the absence of inhibitor; lane 5, hFucTVI wt in COS-7 cells treated with CS and lane 6, hFucTVI wt in COS-7 cells treated with TM.

The α1,3-fucosyltransferase activity in COS-7 cells treated with TM or CS is presented in Figure 3. The activity of wt hFucTV and -VI was completely abolished by the TM treatment, confirming that core glycosylation is required for the expression of hFucTV and -VI enzyme activity. The hFucTV and -VI activity in the CS treated cells was diminished to approximately 50–55% of the activity of the native enzymes and hence trimming of the glucose residues is required for expression of full enzyme activity of hFucTV and -VI. These results demonstrated hFucTV and -VI react in a similar way as hFucTIII [10] to TM and CS treatments.

From the present results, it can be concluded, that in addition to the two C-terminal N-glycosylation sites at least one of the N-terminal potential N-glycosylation sites of wt hFucTV and -VI is glycosylated and that trimming of glucose residues is required for expression of full enzyme activity.

Site-directed mutagenesis

Site-directed mutagenesis was carried out to study the glycosylation of the individual N-terminal potential N-glycosylation sites in hFucTV and -VI (hFucTV: Asn60 and Asn105, hFucTVI: Asn46 and Asn91). Three types of

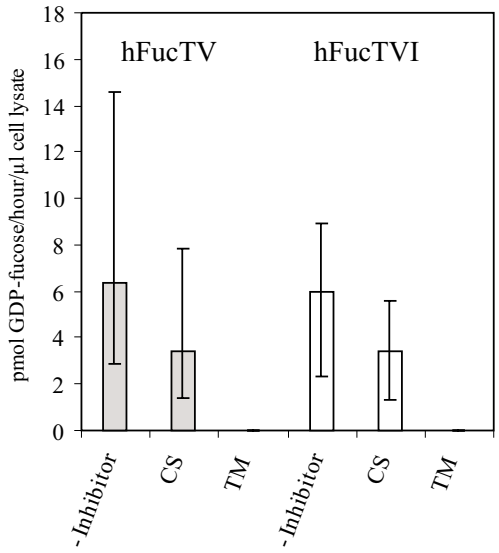

Figure 3. The α1,3-fucosyltransferase activity in lysates of wt hFucTV or -VI transfected COS-7 cells treated with tunicamycin (TM) or castanospermine (CS). *N*-acetyllactosamine was used as oligosaccharide acceptor. The average of four independent experiments is presented for both hFucTV and hFucTVI.

hFucTV and hFucTVI mutants were generated by site-directed mutagenesis (hFucTV: N60Q, N105Q and N60Q/N105Q and hFucTVI: N46Q, N91Q and N46Q/N91Q) (Figures 1B and C). Sequencing of the generated *hFUT5* and *hFUT6* mutants revealed that apart from the mutations present in the target mutagenic primers no other mutations were introduced (data not shown).

Expression level and activity of the mutant hFucTVs and hFucTVIs

The presence of hFucTV or -VI translation product in COS-7 cells transfected with the wt's or one of the six different mutants was verified by Western blotting using the OLI antiserum (Figure 4). Comparable amounts of hFucTV or -VI translation products were detected in all of the transfected COS-7 cells independent of which construct had been used for transfection (Figures 4A, B, lanes 1–4). No hFucTV or -VI translation product was detected in mock-transfected COS-7 cells (Figures 4A, B, lane 5). All the mutants, except hFucTVI N46Q, migrated differently from the wt's according to the number of oligosaccharide chains attached. Wild type hFucTV and -VI migrated at approximately 48 kDa and 44 kDa respectively (Figures 4A, B, lane 1), whereas elimination of Asn60 and Asn105 (hFucTV) and Asn91 (hFucTVI) resulted in a decrease in molecular mass of 2–3 kDa (Figure 4A, lanes 2 and 3 and Figure 4B lane 3). The hFucTVI N46Q mutant migrated at the same position as wt hFucTVI and hence Asn46 is not glycosylated when the native hFucTVI enzyme is expressed in COS-7 cells (Figure 4B, lane 2). The molecular mass of the hFucTV N60Q/N105Q double mutant was decreased with 4–6 kDa corresponding to the size of two N-linked glycans (Figure 4A, lane 4). The hFucTVI double mutant migrated at the same position as the N91Q single mutant (Figure 4B, lane 4), confirming that the Asn46 is not glycosylated. The migration of the upper protein bands at around 97–68 kDa (Figures 4A, B, lanes 1–4) changes in a similar way to the lower hFucTV and -VI bands, according to the number of attached N-linked glycans. This further supports that these bands represent hFucTV and -VI dimers. From these analyses, it was concluded that both of the N-terminal potential N-glycosylation sites of hFucTV are glycosylated, whereas in hFucTVI only one of the N-terminal potential N-glycosylation sites (Asn91) is glycosylated. Combining the results in the present study with our previously obtained results [10] demonstrates that all the potential N-glycosylation sites of hFucTV are glycosylated when hFucTV is expressed in COS-7 cells. Furthermore, only three of the four N-glycosylation sites in hFucTVI are glycosylated when hFucTVI wt is expressed in COS-7 cells. The above results are in accordance with the ~11 and ~9 kDa decrease in the molecular mass of hFucTV and -VI respectively in tunicamycin treated cells (Figure 2). However, the demonstrated glycosylation of Asn105 of hFucTV contrasts with a previous finding, which noted that Asn105 was not glycosylated when hFucTV was expressed in COS-7 cells [12]. In that study, truncated hFucTV wt and mutants (amino acid 76–374) were expressed with an N-terminal protein A, IgG binding domain and not as a full-length native protein as in the present study. This might explain the different glycosylation patterns of hFucTV in the two studies. The α1,3-fucosyltransferase activity of wt hFucTV and -VI and the mutants was measured using *N*-acetyllactosamine as oligosaccharide acceptor (Figure 5). All the single mutants exhibited enzyme activities that did not differ considerably from the activities of wt hFucTV and -VI. These results confirm the previous analyses of the enzyme activity of hFucTV and -VI lacking either Asn105 (hFucTV), Asn46 or Asn91 (hFucTVI) [12,13] and further demonstrate that elimination of Asn60 only leads to a minor decrease in the catalytic activity of hFucTV. In addition, elimination of both potential N-glycosylation sites in hFucTVI (N46Q/N91Q) did not change its enzyme activity

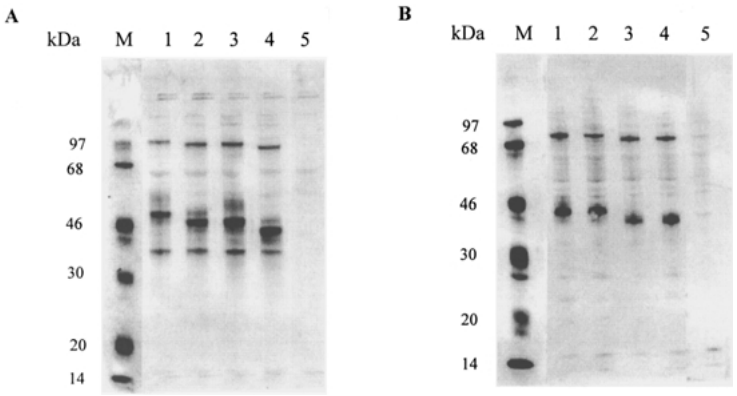

Figure 4. SDS-PAGE and Western blotting analysis of hFucTV (A) and hFucTVI (B) wt's and mutants. A) Lane M, ECL molecular mass markers; lane 1, hFucTV wt cells; lane 2, N60Q; lane 3, N105Q; lane 4, N60Q/N105Q and lane 5, mock transfected COS-7 cells. B) Lane M, ECL molecular mass markers; lane 1, hFucTVI wt cells; lane 2, N46Q; lane 3, N91Q; lane 4, N46Q/N91Q and lane 5, mock transfected COS-7 cells.

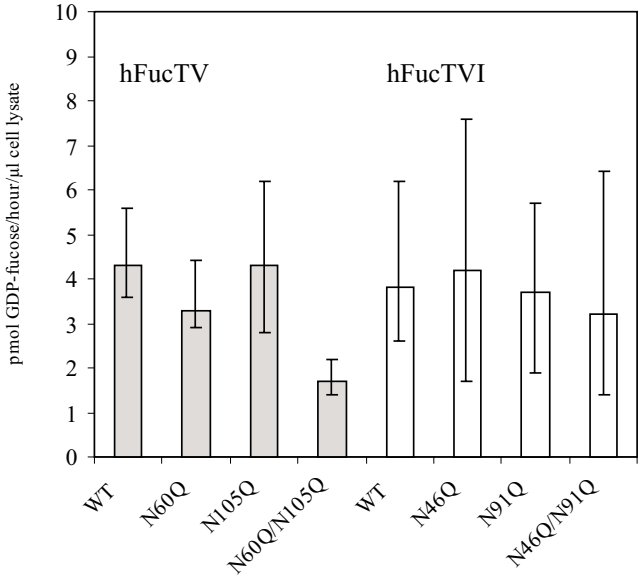

Figure 5. The α1,3-fucosyltransferase activity in the lysates of COS-7 cells expressing either wt hFucTV or -VI, or the mutants. *N*-acetyllactosamine was used as oligosaccharide acceptor. The average of six different experiments is presented.

significantly, whereas abolishment of both sites in hFucTV reduced the enzyme activity to approximately 40% of the wt activity. Hence glycosylation of one of the N-terminal N-glycosylation sites in hFucTV is indispensable for expression of full enzyme activity. No α1,3-fucosyltransferase activity was detectable in mock-transfected COS-7 cells (data not shown).

Although the individual glycosylation of the N-terminal N-glycosylation sites in hFucTV and -VI is not important for the expression of enzyme activity, as demonstrated in the present study, glycosylation of these sites might be involved in the cellular localisation of the two enzymes.

Conclusions

In the present study, we have demonstrated that the two N-terminal potential N-glycosylation sites in hFucTV and one of the N-terminal sites in hFucTVI are glycosylated when the two enzymes are expressed in COS-7 cells. Their individual glycosylation does not contribute considerably to expression of the enzyme activity of the two enzymes. However, in hFucTV one of the N-terminal sites must be glycosylated to obtain an enzyme activity comparable to that of the hFucTV wt. In addition, it was demonstrated that trimming of N-linked glycans of hFucTV and -VI is necessary for the expression of full enzyme activity.

Acknowledgements

We are especially grateful to Susanne Bruun and Inge Lis Thorsen for skillful technical assistance. We thank Professor Eric G. Berger for the generous gift of the OLI antiserum. This work was supported by grants from The Research Initiative of Aarhus Council, The University of Aarhus, Frits, Georg og Marie Cecilie Glud's Legat, MC og JK Moltums fond and Købmand M Kristjan Kjær og Hustru Margrethe Kjær født La Cour-Holmens Fond.

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Received 22 December 2000, revised 12 April 2001,
accepted 18 April 2001