N-Glycosylations of human α1,3-fucosyltransferase IX are required for full enzyme activity

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Human α1,3-fucosyltransferase IX catalyzes the transfer of L-fucose from guanosine diphosphate-β1,1-fucose to N-acetyllactosamine, generating a Lewis X epitope, and is thereby involved in the synthesis of fucosylated cell surface glycoconjugates. It contains three putative N-glycosylation sites (Asn62, Asn101 and Asn153). The current study considers the functional role of these potential N-glycosylations within the enzyme. We produced truncated variants of human fucosyltransferase IX containing the soluble extracellular catalytic domain. To analyze the relevance of each N-glycosylation site, several genomic mutant DNAs encoding a glutamine (Gln/Q) instead of the asparagine residue were created and expressed in Spodoptera frugiperda cells applying a baculovirus expression system. After production and purification of these variants of human FucT IX, the wild-type (wt) enzyme and the variants were characterized regarding their activity and kinetic properties. The variants showed lower activity than the wt FucT, whereas the individual N-glycosylation sites had different effects on the enzyme activity and kinetic parameters. While the single variant N62Q still showed ∼60% of wt activity and N101Q retained ∼30% activity, replacement of Asn153 by glutamine led to an almost complete loss of enzymatic activity. The same could be observed for variants missing two or more putative N-glycosylation sites, which indicated the importance of N-glycosylation for enzyme stability and activity.

Keywords: enzyme kinetics / fucosyltransferases / Lewis X / N-glycosylation / site-directed mutagenesis

Introduction

Glycosylation is one of the most complex types of posttranslational protein modification (Nothaft and Szymanski 2010). Two out of three eukaryotic proteins are glycosylated (Apweiler et al. 1999). Glycans are involved in a number of biological processes, e.g. cell–cell recognition, signal transduction, endocytosis, cellular adhesion or tumor metastasis (Ohtsubo and Marth 2006; Li et al. 2010; Reis et al. 2010). To date, >90 distinct glycosyltransferase (GT) families are known, which catalyze the formation of glycosidic bonds under either retention or inversion of the configuration of the anomeric carbon of the donor substrate (Bourne and Henriass 2001; Coutinho et al. 2003).

One class of inverting GTs, which catalyzes the formation of fucosylated cell surface glycoconjugates, is represented by fucosyltransferases (FucTs). To date, eight human α1,3 fucosyltransferase genes (FUT) have been identified: FUT3—FUT7 and FUT9—FUT11. In contrast to the FUT3—FUT7 genes, the FUT9 gene is evolutionarily highly conserved between mice and humans (Kaneko et al. 1999a). The classical FucTs FucTIII—FucTVII and FucTIX are encoded by monoxenic genes while the recently identified enzymes FucTX and FucTXI are encoded by polyoxenic genes (Mollicone et al. 2009).

The produced glycoconjugates play an important role in embryogenesis, cell adhesion and the development of blood group antigens such as Lewis X (Solter and Knowles 1978; Schonlau and Mai 1995; Ashwell and Mai 1997; Nishihara et al. 2003; Kudo et al. 2007). The enzyme most responsible for the synthesis of Lewis X in the brain is probably α1,3-FucTIX (Nishihara et al. 2003), as it preferentially fucosylates the terminal N-acetyllactosamine in a polylactosamine chain, whereas other known FucTs such as FucTIII—VII prefer the inner polylactosamine chain as acceptor. The human FUT9 gene is localized at 6q16, the long arm of human chromosome 6 (Kaneko et al. 1999b). There are two known transcript sizes, which show a tissue-specific distribution (Cailleau-Thomas et al. 2000). FUT9 is mainly expressed in the central nervous system, in both developing and mature brain of humans, rats and mice (Kaneko et al. 1999a; Baboval et al. 2000; Cailleau-Thomas et al. 2000). The human FucTIX is located in the Golgi and trans-Golgi network (Brito et al. 2008). Like all known human α1,3-FucTs, FucTIX consists of a short cytoplasmic N-terminus, a single membrane spanning region, a lumenally oriented C-terminus with a stem region and the large catalytic domain (Ma et al. 2003). Secreted soluble forms are produced by proteolysis in the Golgi apparatus at multiple
protease sensitive sites within the stem region of the enzyme (Staudacher 1996).

Human $\alpha_1,3$-FucTs show a high degree of sequence similarity at amino acid level for the catalytic domain (Martin et al. 1997; Kaneko et al. 1999a). Martin et al. identified a highly conserved consensus motif that is unique to members of the $\alpha_1,3$-FucT family and was also described by Breton et al. (1998) as motif II. Cloning of human FucTIX revealed the presence of this short conserved peptide motif YxFxL/VxFENS/TxxxxYxTEK within the proposed catalytic domain (Kaneko et al. 1999a). Mollicone et al. later defined this $\alpha_1,3$-FucT motif as a part of motif V, which is altogether with motif IV involved in the donor recognition and binding (Dupuy et al. 2004). Additionally, motif IV contains a potential N-glycosylation site (Christensen, Jensen et al. 2000). In contrast, motifs I–III take part in recognition of the acceptor substrate (Legault et al. 1995). The crystal structure of a truncated Lewis-type FucT from Helicobacter pylori confirmed these findings (Sun et al. 2007). Furthermore, Both et al. (2011) revealed a new “first cluster motif” between the actual motifs IV and V shared by both core and Lewis-type $\alpha_1,3$-FucTs.

Human FucTIX is responsible for the synthesis of Lewis X on various cell types and tissues, e.g. on the lectin dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), which is presented on human granulocytes and peripheral leukocytes (Nakayama et al. 2001; Bogoevska et al. 2006). Other known binding partners for Lewis X, besides Lewis X itself, are selectins, c-type lectins and growth factors, indicating that Lewis X plays an essential role in various recognition processes (Ma et al. 2006). Human $\alpha_1,3$-FucTs themselves are enzymes whose maturation depends on posttranslational modification. However, they differ in their number of putative N-glycosylation sites. While FucTIII possesses only two potential N-glycosylation sites in the catalytic domain, the related FucTs V and VI contain four. Recently, it was shown that especially N-glycosylations in the C-terminal region are essential for full enzyme activity (Christensen, Bross et al. 2000; Christensen, Jensen et al. 2000; Morais et al. 2003). It was demonstrated that lack of N-glycosylation did not affect $K_m$ for both donor and acceptor substrates in FucTIII (Christensen, Jensen et al. 2000). Furthermore, the N-terminal N-glycosylation sites Asn105 of FucTV and Asn46 and Asn91 of FucTVI do not need to be glycosylated for full enzyme activity (Nguyen et al. 1998; Christensen, Bross et al. 2000). Other studies of GTs validated the involvement of N-glycans in protein folding as well as intracellular localization and prevention of proteolytic degradation (Haraguchi et al. 1995; Nagai et al. 1997).

FucTIX, however, comprises three potential N-glycosylation sites at Asn62, Asn101 and Asn153 (Figure 1A), but none in...
the C-terminal region. In the present study, we investigated these putative N-glycosylation sites and their impact on the enzyme activity. Therefore, we created a His-tagged, truncated, soluble variant of human α1,3-FucTIX (HF9) containing a β-trace signal peptide to enable secretion of the enzyme. After purification and basic characterization of the recombinant HF9, we used site-directed mutagenesis to produce HF9 variants lacking one (Figure 1B) or two (Figure 1C) N-glycosylation sites. The study shows that all of these putative sites were N-glycosylated after production of the wild-type (wt) enzyme in insect cells, and elimination of the N-glycosylation sites led to a corresponding reduction of the mass of the enzymes. Additionally, the individual N-glycosylations had different effects on the α1,3-fucosyltransferase activity.

Results
Production and basic characterization of recombinant hFucTIX
In order to obtain high amounts of soluble human FucTIX, we cloned the recombinant variant HF9 (Figure 1A). HF9 includes among others a β-trace secretion signal peptide for secretion of the protein into the cell culture supernatant and a deca-histidine tag for purification. We produced the HF9 variants applying a baculovirus system in Sf9 cells to enable posttranslational modifications. Collection of cell culture supernatant 1–4 days postinfection revealed production of HF9 wt in infected, but not in control Sf9 cells, as shown by western blot analysis (Figure 2A). One to four days postinfection, secreted HF9 wt (~41 kDa) could be detected in different amounts using an anti-FucTIX antibody. Determination of the enzymatic activity in the cell culture supernatant resulted in a maximal activity of 500 mU/L 2 days postinfection (Figure 2B). However, starting at 3 days postinfection, additional signals in western blot analyses were detected. Therefore, an optimal production time of 2 days for HF9 wt was chosen. Purification of HF9 wt from cell culture supernatant was carried out using nickel affinity chromatography and yielded in ~100 µg of pure HF9 from 200 mL of cell culture supernatant (Supplementary data, Figure S1). The purified HF9 exhibited a specific activity of ~250 U/g HF9 at 37°C (Table I).

Site-directed mutagenesis and protein production
HF9 contains three potential N-glycosylation sites (Asn-X-Ser/Thr, X = any amino acid except proline) at amino acid positions 62, 101 and 153, respectively. To create HF9 variants lacking one or two N-glycosylation sites, megaprimer PCR of whole plasmid (Miyazaki 2003) was used in order to replace the asparagine (Asn/N) codons of the putative N-glycosylation sites by glutamine codons (Figure 1B and C). Sequence analyses of the pFastTHF9 mutant plasmids confirmed success of the mutagenesis. Three types of single and three types of double mutants were created resulting in the HF9 variants N62Q, N101Q and N153Q as well as N62Q/N101Q, N62Q/N153Q and N101Q/N153Q. After production and purification of the particular variants, western blot analyses revealed a decrease in the molecular weight of ~2 kDa for each missing N-glycosylation site (Figure 3). The apparent molecular mass for wt HF9 was 41 kDa, variants lacking one N-glycosylation showed an apparent mass of ~39 kDa and those missing two N-glycosylation sites only had ~37 kDa.

Table I. Specific activity of HF9 variants

<table>
<thead>
<tr>
<th>HF9 variant</th>
<th>Yield HF9 [µg/mL]</th>
<th>Specific activity [U/g]</th>
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</thead>
<tbody>
<tr>
<td>wt</td>
<td>9.6</td>
<td>248 ± 1</td>
</tr>
<tr>
<td>N62Q</td>
<td>40</td>
<td>154 ± 12</td>
</tr>
<tr>
<td>N101Q</td>
<td>9.1</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>N153Q</td>
<td>1.9</td>
<td>&lt;10</td>
</tr>
<tr>
<td>N62Q/N101Q</td>
<td>1.8</td>
<td>&lt;10</td>
</tr>
<tr>
<td>N62Q/N153Q</td>
<td>1.7</td>
<td>&lt;10</td>
</tr>
<tr>
<td>N101Q/N153Q</td>
<td>1.8</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Fig. 2. Time course of HF9 wt production in baculovirus infected and control Sf9 cells. Cell culture supernatant was collected one to 4 days postinfection and analyzed via western blotting (A). Activity in cell culture supernatants was examined using standard reaction conditions (B). Uninfected cells were used as control. The average of three independent experiments is presented.

Fig. 3. Western blot analysis of HF9 variants after production in baculovirus infected Sf9 cells and purification via nickel affinity chromatography. HF9 wt, 1; N62Q, 2; N62Q/N101Q, 3; cell culture supernatant of cells infected with baculovirus in the presence of tunicamycin, 4; cell culture supernatant of uninfected cells, 5.
This distinction corresponded to the mass of an N-glycan produced in insect cells, which is 1.88 kDa resulting from Man9GlcNAc2 (Altmann et al. 1999). Treatment of infected Sf9 cells with tunicamycin, which inhibits biosynthesis of N-glycans (Elbein 1984), led to HF9 lacking all the three N-glycosylations. No detectable FucTIX was produced within Sf9 control cells. In addition, the quantity of secreted and purified enzyme varied depending on the position of the missing N-glycosylation. Whereas the variants N62Q and N101Q could be produced in amounts comparable with those of the wt HF9, variants lacking the N-glycosylation at amino acid position 153 or >1 N-glycosylation site only gave very low protein yields (Table I). However, it could be shown that considerable amounts of those latter variants remained within the cells and were not secreted (Supplementary data, Figure S2).

Effect of N-glycosylation on enzyme activity
Measurements of α1,3-fucosyltransferase activity of the purified enzymes revealed a diminished activity compared with HF9 wt for each of the variants. While the variant N62Q still showed ~62% of the wt HF9 activity, lack of N-glycosylation at position 101 led to a reduction of the specific activity to 67 U/g and thereby to 27% (Figure 4). The other variants, including N153Q and each of the double variants, did not exhibit any remarkable α1,3-fucosyltransferase activity anymore, neither in the cell culture supernatant of baculoviral infected cells nor for the purified proteins. The detected specific activities did not exceed values of 10 U/g, which corresponded to <5% of the wt HF9 activity, demonstrating that N-glycosylation at Asn153 is more essential for full enzyme activity than the ones at Asn62 and Asn101, respectively. In addition, abolishment of two N-glycosylation sites caused in each case an almost complete loss of α1,3-fucosyltransferase activity.

Kinetic analyses of active HF9 variants
Furthermore, the active variants N62Q, N101Q and HF9 wt were characterized regarding their kinetic parameters. \( K_M \) values for both donor and acceptor substrates were obtained by one site binding analysis of the resulting curves (Supplementary data, Figure S3). As shown in the Lineweaver–Burk plot (Figure 5A and B), the apparent \( K_M \) for both the donor guanosine diphosphate (GDP)-fucose and the acceptor N-acetyllactosamine differed slightly from the HF9 wt. While the \( K_M \) of wt HF9 was 0.61 mM for N-acetyllactosamine, the corresponding \( K_M \) values of the active variants were reduced to 0.27 mM for N62Q and 0.22 mM for N101Q, respectively. In contrast, \( K_M \) for GDP-fucose was slightly enhanced for both variants in comparison with HF9 wt, for which it amounted to 2.6 µM. The \( K_M \) values turned out to be 6.6 µM for N62Q and 4.7 µM for N101Q. The minor differences in these values indicated that missing N-glycosylations did influence the affinity for both acceptor and donor substrates, but the differences were not in a significant range. Turnover numbers for both N62Q and N101Q were reduced compared with HF9 wt, with \( K_{cat} \) values of 7.1 and 2.5 min\(^{-1}\), respectively (for further kinetic data see also Fig. 4.

Fig. 4. Specific activity of HF9 variants. α1,3-fucosyltransferase activity was examined using standard reaction conditions. N-acetyllactosamine was applied as acceptor and GDP-β-L-fucose as donor substrates (Materials and methods). The average of three independent experiments is presented.

Fig. 5. Lineweaver–Burk analyses of the active variants HF9 wt (squares), N62Q (triangles) and N101Q (dots). Kinetic analyses were performed using 50–200 ng of the particular enzyme and various concentrations of either GDP-β-L-fucose (A) or N-acetyllactosamine (B) under otherwise standard reaction conditions until a maximum of 20% conversion of GDP-β-L-fucose. The average of three independent experiments is presented.
Supplementary data, Table SI), validating the loss of efficiency for the HF9 variants lacking N-glycosylations.

Discussion

Production and basic characterization of recombinant hFucTIX

In recent studies, a soluble truncated form of HF9 was produced constitutively in insect cells (Brito et al. 2007). However, α1,3-FucTIX activity was only monitored from the cell culture supernatant and, to our knowledge, the fully glycosylated enzyme had not been purified so far. Recombinant production of human FucTs such as α1,3-FucTVII (Shinkai et al. 1997), α1,3/4-FucTIII (Morais et al. 2001) and α1,6-FucTVIII (Ihara et al. 2006) using a baculovirus system was already implemented before and gave high yields of the respective enzyme. Recently, our laboratory published the comparison of different expression systems for α1,3-FucTIX and found both the constitutive and baculovirus-induced approach suitable for the production of high amounts of soluble enzyme (Stacke et al. 2010). Within the first part of the current work we cloned, produced and purified HF9, a soluble truncated variant of HF9, as well as its N-glycosylation variants. The applied baculovirus expression system proved to be suitable for the production of recombinant HF9. Examination of α1,3-fucosyltransferase activity in the cell culture supernatant 2 day postinfection revealed an activity of 500 mU/L, which is 34-fold higher than the one reported by Brito et al. (2007). Multiple bands in western blot analyses, probably due to heterogeneity in the glycosylation pattern in insect cells (Morais et al. 2003), could be observed in cell culture supernatant 3 days postinfection. Loss of FucT activity in the cell culture supernatant did not correlate with the apparently higher enzyme amounts. However, a few days postinfection the baculovirus induced lysis of cells starts, which might lead to a release of inactive protein and also possible small molecule inhibitors of the enzyme, diminishing FucT activity.

Purification via deca-histidine tag gave ~25% yield of the HF9 that was initially present in the cell culture supernatant. This value is comparable with the 28% yield for α1,6-FucTVIII published by Ihara et al. (2006) and significantly higher than a total yield of 2% achieved, e.g. by purification of full length FucTIII from baby hamster kidney cells (Sousa et al. 2001). The specific activity detected for HF9 was ~100-fold higher than the one detected for rat α1,3-FucTIX (Baboval et al. 2000), which is highly homologous to HF9.

Site-directed mutagenesis and protein production

Detailed examination of the effect of N-glycosylation on the α1,3-FucTIX activity was never accomplished before. However, Brito et al. (2007) revealed that each of the three potential N-glycosylation sites in α1,3-FucTIX was indeed glycosylated. We could confirm this finding by tunicamycin treatment, which led to a decrease in the molecular weight of ~6 kDa in comparison with HF9 wt due to the lack of all the three N-glycosylation sites. Based on this knowledge, six genomic mutants were created that encoded HF9 variants lacking one or two potential N-glycosylations sites. The production and purification of these variants could be implemented in the same manner as for HF9 wt. However, the low yields detected for the variants missing two N-glycosylation sites or the N-glycosylation at Asn153, respectively, correlate with the high amounts of protein retaining inside the cells, and not being secreted.

Effect of N-glycosylation on enzyme activity

Recent studies of human FucTIII, -V and -VI (Christensen, Bross et al. 2000; Christensen, Jensen et al. 2000) revealed the necessity of the C-terminal N-glycosylation sites for expression of full enzyme activity. In contrast, the two conserved N-glycosylation sites in human FucTV and -VI do not contribute to the enzyme activity individually. We could demonstrate that each of the N-glycosylation sites in HF9 contributed to full enzyme activity. Within HF9, however, the individual sites differed in their effect on the enzyme activity. Unique replacement of the two N-terminal N-glycosylation sites Asn62 or Asn101 also led to a decrease in the specific activity, and the HF9 variant N153Q did not show any FucT activity at all. Whereas the latter is in accordance with the findings for other human α1,3-FucTs such as FucTV, the former is a novel discovery for this enzyme class. This might be due to the fact that the potential N-glycosylation sites Asn62 and Asn101 in human FucTIX are relatively close to the conserved peptide motifs I and II, respectively, whereas in FucTV and -VI these sites are separated from the mentioned motifs by >15 amino acids (Christensen, Jensen et al. 2000; Mollicone et al. 2009). Our results demonstrate that N-glycosylation at least at Asn153 is necessary to gain active enzyme. Asn153, however, is situated at the beginning of motif IV, as well as the corresponding N-glycosylation site in FucTIII, -V and -VI. Deletion of this N-glycosylation led to the loss of FucT activity, which is probably due to its location within this conserved motif. In addition, absence of >1 N-glycosylation caused not only low yields but also almost no detectable FucT activity, whereas it did not matter which two out of the three N-glycosylation sites had been varied. This is consistent with former studies that revealed the importance of glycosylation for proper folding and N-linked oligosaccharides playing multiple roles during the conformational maturation of glycoproteins, e.g. the stabilization of folded domains (Helenius 1994).

Kinetic analyses of HF9 active variants

We here report for the first time studies of the influence of N-glycosylations on the kinetic parameters of human FucTIX. Slight changes were detected for both $K_M$ values, comprising a decrease in the one for $N$-acetyllactosamine and an increase in that for GDP-fucose. These values indicated a small but not significant change in the affinity for both acceptor and donor substrates. As both the N-glycosylation site Asn62 and Asn101 are approximate to motifs I and II, respectively, the slight decrease in the $K_M$ values could be explained by this change in these acceptor-binding motifs. However, those decreases in $K_M$ were only relatively small. This is in agreement with the results of Christensen, Jensen et al. (2000), who could demonstrate that the lack of the N-glycosylation site at N185 in hFucTIII did affect the affinity neither for the oligosaccharide acceptor nor toward the nucleotide sugar.
To the best of our knowledge, detailed kinetic studies of other human α1,3-FucTs and their N-glycosylation variants, respectively, did not exist so far. Although the importance of N-glycosylation for enzyme activity is obvious, the oligosaccharide chains did not seem to be involved in the substrate interaction, pointing to the fact that N-glycosylations might be a prerequisite for the proper folding into a catalytically active enzyme. Of course, the effect of each missing N-glycan could vary between insect and human cell derived enzymes due to their different glycosylation patterns. Although insect cells are capable of transferring glycans to the same sites as used for N-glycosylation in mammalian cells, glycans in mammalian cells are more complex containing terminal sialic acids, whereas insect glycans terminate in mannose residues due to the lack of appropriate enzymes (Harrison and Jarvis 2006). Nevertheless, insect cells are suitable for the production of recombinant proteins, if not yet ideal for the production of pharmaceutical-quality glycoproteins as core-fucosylation can induce allergic reactions (Tomiya et al. 2004). The here-presented application of a baculovirus system in insect cells to produce active FucT, however, provides insight into the effects of the individual N-glycosylation sites on the HF9 activity.

**Conclusion**

The current study demonstrates the effect of N-glycosylation in HF9 on the enzyme activity. This effect was investigated by production and purification as well as enzymatic characterization of recombinant soluble HF9 variants. We were the first to demonstrate the importance of each of the N-glycosylation sites for full human FucT IX activity, depending on their proximity to different acceptor as well as donor binding motifs.

**Material and methods**

### Chemicals and solvents

If not mentioned otherwise, all chemicals and solvents were obtained from Sigma-Aldrich (München, Germany), Roth (Karlsruhe, Germany), Calbiochem (Darmstadt, Germany) or AppliChem (Darmstadt, Germany).

### Cloning of HF9 gene containing vector pFastTHF9

To enable bacmid preparation and constitutive production of HF9 in insect cells, the vector pFastTHF9 was generated via several cloning steps. First, the vector pIB/Mel-opt-H10 (Grunwald et al. 2006), a vector based on pIB/V5-His (Invitrogen, Darmstadt, Germany), was modified. To create new restriction sites, the annealed product of primers PZ1 (all primers are listed in Table II) and PZ2 was introduced into pIB/Mel-opt-H10 between the XhoI and SacII restriction sites. In a second step, the annealed product of primers PZ3 and PZ4 was inserted between the SalI and NdeI sites resulting in the vector pMelHis(+). The polymerase chain reaction (PCR) product using the primers CS10 and CS11 with pEF-BOS-FUT9 (Bogoevska et al. 2006) as template was cloned into pMelHis(+) using XhoI and NdeI, yielding pMHF9. The β-trace signal peptide coding sequence was amplified using primers CS13 and CS14 with pIB-FUT5 as template (Munster et al. 2006). The PCR product was inserted via HindIII and

### Table II. Primers applied in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
</tr>
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<tbody>
<tr>
<td>General</td>
<td>pFastBacHTa rev</td>
</tr>
<tr>
<td>polyhedrin fw</td>
<td>AAA TGA TAA CCATCT CGC</td>
</tr>
<tr>
<td>N62Q N62Q fw</td>
<td>ACT GAT TAT TTT CAG GAA ACT ACT ATT</td>
</tr>
<tr>
<td>N101Q N101Q fw</td>
<td>AAA ACT GAT TAT TTT CAG</td>
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<tr>
<td>R111A R111A fw</td>
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<tr>
<td>D112A D112A fw</td>
<td>CAT CAC CGA GCC ATG TTG G</td>
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<td>Cloning of pFastTHF9</td>
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<tr>
<td>PZ1</td>
<td>TCG AAA TCG AGG GAG GGG TGCT ACT CGA TCG ATA TGAG TAC CGC GG</td>
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<td>PZ2</td>
<td>GGT ACC ATA TCG ATC CTC GAG ACC CTT TCG ACT GAT</td>
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<td>PZ3</td>
<td>tcg aCT CGA GAA TTC GCT AGC GCC GGC GAG GTC A</td>
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<td>PZ4</td>
<td>TAT GAG CTC GCC GCC GCT AGC GAA TTC TCG AG</td>
</tr>
<tr>
<td>CS10</td>
<td>C GAC TCG AGA AGT CGA ATG GAA TCG TCA GCC AGC</td>
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<tr>
<td>CS11</td>
<td>AT TAC CAT ATG TTA ATT CCA AAA CCA TTAT CTC</td>
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<td>CS13</td>
<td>TAG AAG CTG ACT CAT CAC AGC CTG</td>
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<tr>
<td>CS16</td>
<td>A GCT GAA TCC AGG CCT TCT AGA</td>
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BamHI sites into pMHF9 to replace the melittin signal peptide with the β-trace signal peptide gene, thus generating the plasmid pTHF9. Additional restriction sites were introduced in the vector pTHF9 by inserting the annealed product of primers CS15 and CS16 at the HindIII site yielding pTHF9 + ESX. Finally, pTHF9 + ESX and pFastBac1T were digested with EcoRI and KpnI and ligated to obtain the shuttle vector pFastTHF9.

The resulting vector encoded a β-trace secretion signal, a deca-histidine tag and a factor Xa protease cleavage site located at the N-terminus of hS39F9, a truncated form of human FucT IX lacking the N-terminal amino acids up to Serine39 (trans-membrane region) but containing the catalytic domain (HF9). The obtained plasmids were verified by sequencing.

Cloning of pFastTHF9 mutants

Mutants of pFastTHF9 resulting in HF9 variants were created using Megaprimer PCR of whole plasmid (Miyazaki 2003). In the first PCR, the particular megaprimer was created using forward primers carrying the mutation (listed in Table II) and pFastBacHT rev as reverse primer. Phusion HF DNA polymerase (ThermoScientific, Bonn, Germany) was used with its corresponding buffer. After an initial denaturing step at 98°C for 2 min, the following cycle conditions were repeated 30 times: 98°C (30 s), 45°C (60 s) and 72°C (45 s). The resulting megaprimer were purified via PCR Cleanup and Gel Extraction Kit (Sued-Laborbedarf GmbH, Gauting, Germany). Megaprimer PCR of whole plasmid was carried out according to literature (Miyazaki 2003). After restriction with DpnI for 1 h, transformation of Escherichia coli Top10 cells was performed using 5 µL of the mixture. The cells were plated on Lysogeny Broth agar containing ampicillin and positive clones selected via PCR screening (primers listed in Table II).

To generate the recombinant bacmid bacTHF9 and its mutants, E. coli DH10Bac cells were transformed with the particular pFastTHF9 vector. Transposition proceeded between the mini-Tn7 element on the vector and the mini-attTn7 target site on the bacmid DNA resulting in bacTHF9. Isolation of recombinant bacmid DNA from DH10Bac cells was performed as described by Invitrogen (LifeTechnologies 2004).

Cell culture

Sf9 cells (Invitrogen, Darmstadt, Germany) were cultivated in Insect XPRESS medium (Lonza, Cologne, Germany) or HyClone SFX-Insect Medium (ThermoScientific, Bonn, Germany) containing 10 µg/mL gentamycin (Invitrogen, Darmstadt, Germany) at 27°C as monolayer culture or in suspension at 110 rpm. Subculturing was performed every 3 to 4 days.

Preparation of recombinant baculovirus BacTHF9

Sf9 cells (1×10^6) were transfected with the bacmid DNA bacTHF9 using Insect GeneJuice Transfection reagent according to the manufacturer’s protocol (Novagen, Darmstadt, Germany). After transfection, cells were cultured at 27°C for 48 h. Amplification of viral titer was carried out to gain a high titer virus stock. Tunicamycin-treated cells were infected and 1 µg Tunicamycin per mL was added 4 h postinfection. Cells were harvested after additional 20 h.

Protein production and purification

Production of soluble human FucT IX variant HF9 was performed in Sf9 cells. Optimal multiplicity of infection was detected by infecting 2×10^6 cells with different amounts of baculoviral stock and incubation at 27°C in a 24-well plate, followed by western blot analysis.

Sf9 cells were infected with the determined amount of virus stock and cell culture supernatant was harvested 2 days postinfection. Cell culture supernatant was concentrated using a cross flow filtration system (Quixstand Benchtop System, hollow fiber cartridge UFP-10-C-4X2MA, Nominal MWCO 10 kDa, GE Healthcare, München, Germany). In the meanwhile, buffer was exchanged to nickel nitrilotriacetic acid (Ni-NTA) binding buffer (20 mM Tris, pH 8, 500 mM NaCl, 20 mM imidazole). Purification was performed using an ÄKTAPrime™ plus (GE Healthcare, München, Germany) at 4°C. After equilibration of a 1 mL HisTrap™ FF column (GE Healthcare, München, Germany) with 10 column volumes (cv’s) of Ni-NTA binding buffer, the concentrated protein sample was loaded onto the column via sample loop. Elution of unspecifically bound proteins was performed by increasing imidazole concentration to 30 mM >20 cv. Elution of specifically bound proteins was carried out using 30 cv of Ni-NTA binding buffer containing 500 mM imidazole.

The following dialysis of fractions containing purified HF9 was accomplished overnight at 4°C in dialysis buffer (20 mM Tris, 10 mM NaCl, pH 8.5) and fractions were stored at −20°C after addition of glycerol to a final concentration of 30%.

Analysis of HF9 purity

Success of HF9 purification was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie or silver staining and western blot analysis. Proteins were separated on a 12% SDS polyacrylamide gel (Laemmli 1970). For western blot analysis, proteins were transferred to a polyvinylidene fluoride membrane (Roth, Karlsruhe, Germany) by electrophoresis and blocking was performed with blocking buffer (0.1% bovine serum albumin (BSA) in Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.5)). Immunostaining was carried out using goat anti-FucTIX (1:4.000 in blocking buffer; Santa Cruz Biotechnology, Santa Cruz, CA) as primary and anti-goat-IgG-alkaline phosphatase (1:10.000 in blocking buffer; Sigma-Aldrich, Taufkirchen, Germany) as secondary antibodies. Detection was followed using 1% nitro blue tetrazolium chloride and 5% 5-bromo-4-chloro-3-indolyl phosphate in reaction buffer (100 mM Tris, pH 8.5, 4 mM MgCl2).
saline buffer, the plate was again incubated overnight at 4°C using goat anti-FucTIX (1:4.000 in 0.5× blocking buffer; Santa Cruz Biotechnology, Santa Cruz, CA). Incubation with anti-goat-IgG-alkaline phosphatase (1:10.000 in blocking buffer; Sigma-Aldrich, Taufkirchen, Germany) was carried out for 2 at room temperature. Detection was performed via para-nitrophenylphosphate in reaction buffer (100 mM Tris, pH 8.5, 4 mM MgCl₂).

α1,3-fucosyltransferase IX activity assay
Activity analysis of recombinant HF9 was performed using radioactively labeled GDP-[¹⁴C]-β-L-fucose and GDP-β-L-fucose as donor and N-acetyllactosamine as acceptor substrates. The reaction mixture, containing 1.8 µM GDP-[¹⁴C]-fucose (Perkin Elmer, Rodgau, Germany), 312 µM GDP-β-L-fucose, 4 mM N-acetyllactosamine, 5 mM MnCl₂ and 100 ng–1 µg of purified HF9 in citrate/phosphate buffer, pH 4.5, was incubated at 37°C for 60 min or until a maximum of 20% conversion of GDP-β-L-fucose. Reaction was stopped by adding 375 µL dH₂O and the mixture was filtered via anion exchange chromatography (AG 1-X8 resin, chloride form, BioRad, Münich, Germany). Elution of the reaction product was performed by rinsing the column with 400 µL dH₂O. The resulting radioactively labeled product was analyzed in a liquid scintillation counter (Perkin Elmer, Rodgau, Germany).

All the following experiments regarding enzyme kinetics were carried out for 1 h or until a maximum of 20% conversion of GDP-β-L-fucose. Kinetic data for each N-acetyllactosamine and GDP-β-L-fucose were determined using different concentrations of the particular substrate (0.05–4 mM for N-acetyllactosamine; 0.2–24 µM for GDP-β-L-fucose) under otherwise standard reaction conditions. Furthermore, for GDP-β-L-fucose kinetics, 0.1 µM GDP-[¹⁴C]-β-L-fucose was used instead of 1.8 µM.

Supplementary data
Supplementary data for this article are available online at http://glycob.oxfordjournals.org.

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Conflict of interest
None declared.

Abbreviations
GT, glycosyltransferase; FucTs, fucosyltransferase; FUT, fucosyltransferase genes; PCR, polymerase chain reaction; HF9, human; fucosyltransferase IX; Sf9, Spodoptera frugiperda; cv, column volumes; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Asn/N, asparagine; Gln/Q, glutamine; wt, wild type; His₁₀, deca-histidine tag; Ni-NTA, nickel nitrilotriacetic acid.

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