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Strict order of (Fuc to Asn-linked GlcNAc) fucosyltransferases forming core-difucosylated structures

Erika Staudacher* and Leopold März

Institut für Chemie, Universität für Bodenkultur, Muthgasse 18, A-1190 Vienna, Austria

In insect cells fucose can be either α1,6- or α1,3-linked to the asparagine-bound GlcNAc residue of N-glycans. Difucosylated glycans have also been found. Kinetic studies and acceptor competition experiments demonstrate that two different enzymes are responsible for this α1,6- and α1,3-linkage of fucose. Using dansylated acceptor substrates a strict order of these enzymes can be established for the formation of difucosylated structures. First, the α1,6-fucosyltransferase catalyses the transfer of fucose into α1,6-linkage to the non-fucosylated acceptor and then the α1,3-fucosyltransferase completes the difucosylation.

***Keywords*: fucosyltransferase, insect glycobiology, difucosylation**

***Abbreviations*: Fuc-T C3, GDP-fucose:β-*N*-acetylglucosamine (Fuc to Asn-linked GlcNAc)α1-3-fucosyltransferase; Fuc-T C6, GDP-fucose:β-*N*-acetylglucosamine (Fuc to Asn-linked GlcNAc)α1-6-fucosyltransferase; GlcNAc-transferase I, UDP-GlcNAc:Manα1-3-R[GlcNAc to Manα1-3]β1-2-GlcNAc-transferase I (E.C. 2.4.1.101); GlcNAc-transferase II, UDP-GlcNAc: Manα1-6-R[GlcNAc to Manα1-6]β1-2-GlcNAc- transferase II (E.C. 2.4.1.143); GlcNAc-transferase III, UDP-GlcNAc:R_1-Manα1-6[GlcNAcβ1-2Manα1-3]Manβ1-4-R_2[GlcNAc to Manβ1-4]β1-4-GlcNAc-transferase III (E.C. 2.4.1.144); for acceptor structures see Figure 1.**

Introduction

Compared to the wealth of information available on the structures, biosynthesis and catabolism of N-glycans from mammalian glycoproteins, our knowledge of these glycans from invertebrate sources, in particular insects, is still fragmentary [1]. The application of baculovirus-derived expression systems to insect cell culture technology for the production of proteins and glycoproteins [2, 3] raises the importance of the elucidation of the post-translational modification systems of insect cells, especially their glycosylation capacity with their quite different fucosylation potential [4, 5].

The biochemical pathway processing N-glycans from $Glc_3Man_9GlcNAc_2$ to complex structures is based on a strict order of the action of many glycosidases and glycosyltransferases. In mammalian cells the exact timing of the action of the core α1,6-fucosyltransferase is very well known. It is present in the late Golgi cisternae and transfers the fucose after the action of glucosidases, mannosidases and GlcNAc-transferase I [6]. GlcNAc-transferase II does not preclude the action of α1,6-fucosyltransferase, whereas GlcNAc-transferase III does [7]. This sequential action of the enzymes can be determined by their *in vitro* substrate specificities. So far all known α1,6-fucosyltransferases require an unsubstituted GlcNAc residue linked β1,2 to the α1,3-mannose of the N-glycan [8–10].

Complex glycans derived from plants differ from mammalian cells by the occurrence of a xylose-residue and by the different linkage of the core-fucose. Here fucose is linked α1,3 to the inner GlcNAc. Plant α1,3-fucosyltransferase also needs an unsubstituted GlcNAc residue on the α1,3 arm of the N-glycan [11]. The sequence of fucosylation and xylosylation is still under investigation [12, 13]. However, plant fucosyltransferase does not need a previous xylosylation [11].

In insect cells fucose can be either α1,6- or α1,3-linked to the asparagine bound GlcNAc residue. Difucosylated glycans have also been found [5, 14–16]. Earlier investigations showed that the α1,6- and α1,3-activities in insects also require the unsubstituted terminal GlcNAc on their acceptor substrates.

In the present study we now report that the α1,6- and α1,3-fucosyltransferase activities in *Mamestra brassicae* cells

*To whom correspondence should be addressed. Tel: + 43-1-36006-6063, Fax: + 43-1-36006-6059, e-mail: estaud@edv2.boku.ac.at

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are due to two different enzymes. Furthermore the strict order of these enzymes forming difucosylated structures is shown.

Materials and methods

Materials

GDP-fucose:*β*-*N*-acetylglucosamine (Fuc to Asn-linked GlcNAc) α-1,3-fucosyltransferase (Fuc-T C3) from mung beans was prepared as previously described [11]. GDP-fucose:*β*-*N*-acetylglucosamine (Fuc to Asn-linked GlcNAc) α-1,6-fucosyltransferase (Fuc-T C6) from porcine brain was prepared according to Uozomi *et al.* [10]. Sf-9 cells (from *Spodoptera frugiperda*), a gift from Dr W. Ernst (Institut für angewandte Mikrobiologie, Universität für Bodenkultur, Wien), and MB-0503 cells (from *Mamestra brassicae*), provided by Professor J. Glößl (Institut für angewandte Genetik, Universität für Bodenkultur, Wien), were homogenized and washed as described earlier [4] and diluted with 0.25 M sucrose to a final protein content of 5–10 mg ml^{-1} (Bio-Rad protein assay with bovine serum albumin as a standard).

α-L-Fucosidase from bovine kidney was obtained from Boehringer Mannheim. GDP-L-fucose was purchased from Sigma and GDP-[U-^{14}C]-fucose (specific activity 200–370 mCi mmol^{-1}) was from Amersham. All other materials were purchased in the highest quality available from Merck or from Sigma.

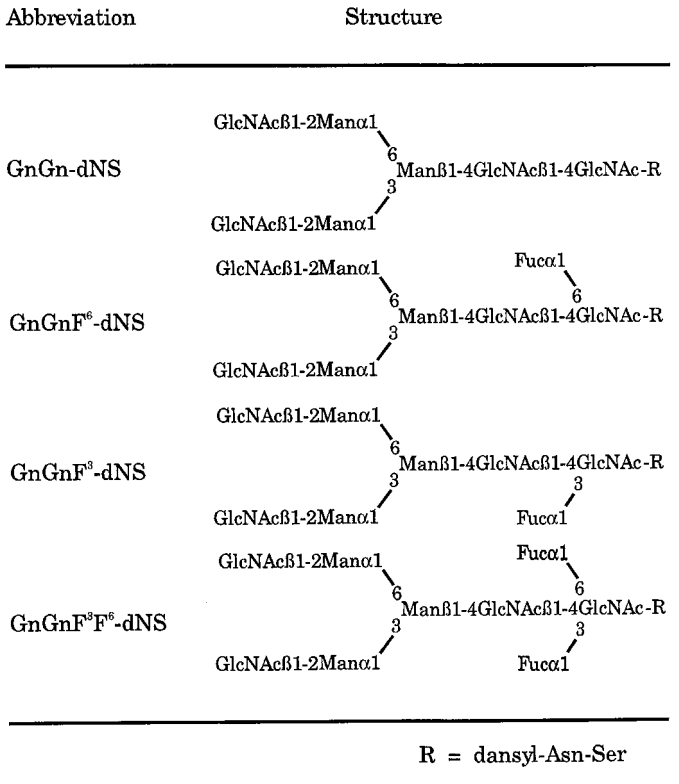

Figure 1. Structures of dansylated glycopeptides with differently fucosylated core-GlcNAc.

Preparation of acceptors

The glycopeptides for the kinetic studies (GnGn-peptide from bovine fibrin and GnGnF^6-peptide from human IgG; for structures see Figure 1) were obtained by extensive digestion with pronase, chemical desialylation and enzymatic degalactosylation as previously described [17]. GnGnF^6-peptide was further purified on lentil lectin to remove non-fucosylated structures [14].

The starting point of all dansylated glycans was GnGnF^6-peptide. The dansylation and preparative separation by reverse phase C18–HPLC were performed as described [18]. GnGn-dNS was obtained by digestion of 50 nmol GnGnF^6-dNS with 10 mU of α-L-fucosidase for 20 h at 37 °C. GnGnF^3-dNS was prepared by incubating GnGn-dNS with Fuc-T C3 from mung beans for 24 h at 37 °C as previously described [11]. Before use, all dansylated acceptors were applied on reverse phase HPLC with the conditions given for the analytical separations (see below). Quantitation was carried out by amino-sugar determination [19].

Assay procedures

Kinetic data were acquired by incubating GnGn- or GnGnF^6-peptide at eight different concentrations ranging from 0.125 to 1.5 mM in a total volume of 20 µl with 1.5 mM GDP-[^{14}C]-fucose (5000 cpm nmol^{-1}), 0.1 M 2-(*N*-morpholino) ethanesulfonic acid/HCl (Mes) at pH 7.0, 0.1% (w/v) Triton X-100, 10 mM $MnCl_2$, 5 mM AMP and 5 µl of MB 0503 cell extract for 4 h at 37 °C. Termination of the reaction, processing over Dowex AG 1 × 8 (Cl^--form) and liquid scinitillation counting was done as previously described [20]. Product formation was calculated after subtraction of control values obtained without acceptor. One unit of activity is defined as the amount of enzyme catalysing the transfer of 1.0 µmol of fucose per min. All assays were carried out at least in duplicate with appropriate controls.

For the preparative transfer of fucose into dansylated acceptors the standard incubation mixture contained, in a total volume of 20 µl, 0.05 mM acceptor (GnGn-dNS, GnGnF^3-dNS or GnGnF^6-dNS), 0.25 mM GDP-fucose, 1–2 µU of mung bean or porcine brain fucosyltransferase or 5 µl of insect cell extract, 0.1 M Mes/HCl at pH 7.0, 0.1% (w/v) Triton X-100 and 5 mM AMP. Based on previously published assay conditions, 10 mM EDTA was added when assaying porcine brain fucosyltransferase and 10 mM $MnCl_2$ was added for mung bean fucosyltransferase or insect cell extracts [4, 10, 11]. Incubations were carried out for 16 h at 37 °C. The reaction was terminated by heating at 100 °C for 3 min. After centrifugation at 13 000 × **g** for 10 min, the supernatant was used directly for HPLC-analysis.

Analytical HPLC-separations

Separations were achieved by using 0.05 M potassium phosphate, pH 2.0, as buffer A and 70% (v/v) acetonitrile in water as buffer B under isocratic elution with 11% B at a flow rate of 0.6 ml min^{-1} for a 3×250 mm 5 µ Hypersil ODS column [21]. The dansylated glycopeptides were detected at Ex/Em 315/550 nm by their fluorescence.

Analytical α-L-fucosidase digest

Incubations were performed with 0.1 nmol of dansylated glycopeptide and 0.1 or 10 mU of α-L-fucosidase from bovine kidney in the presence of 50 mM sodium citrate pH 4.0 for 20 h at 37 °C.

Results and discussion

In insect cells mono- and difucosylated structures have been found with fucoses linked α1,3 or/and α1,6 to the inner GlcNAc residue of N-glycans [5, 14–16]. GnGn-peptide was a suitable acceptor for both corresponding fucosyltransferase activities [4].

Determination of kinetic parameters

In this study GnGn- and $GnGnF^6$-peptides were used as acceptors for the determination of the K_m and V_{max} values of the fucosyltransferase activities in MB 0503 cell extract. The apparent K_m and V_{max} values were evaluated from the initial rate data, using Lineweaver-Burk plots, to be 0.82 mM and 0.188 mU mg^{-1} protein respectively, using GnGn-peptide as acceptor substrate, and 0.40 mM and 0.125 mU mg^{-1} protein using $GnGnF^6$-peptide (Figure 2).

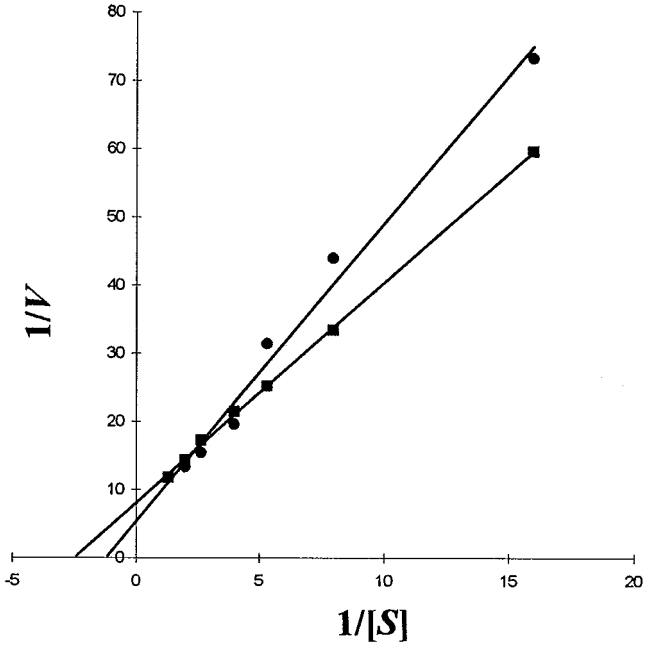

Figure 2. Lineweaver-Burk plots of the MB 0503 α-L-fucosyltransferases using GnGn-peptide (●–●) and $GnGnF^6$-peptide (■–■) as acceptors. Velocity (V) is calculated as mU mg^{-1} protein while the acceptor concentrations (S) are in mM. All conditions are described in Materials and methods.

Table 1. Competition experiments. Enzyme rates were measured as described for the kinetic experiments. Calculated values are obtained for two enzymes (2E) as the sum of the activities for separate incubations with only a single substrate; for one enzyme (1E) theoretical values were calculated according to Dixon and Webb [22]: $v = [V_1(S_1/K_1) + V_2(S_2/K_2)]/[1 + S_1/K_1 + S_2/K_2]$, where S_1 and S_2 are the concentrations of the two substrates, V_1 and V_2 are the V_{max} values and K_1 and K_2 are the K_m values.

Substrate concentrations (mM)		*Fucosyltransferase activity (mU mg^{-1} protein)*		
GnGn-peptide	*$GnGnF^6$-peptide*	*Experimental*	*Calculated*	
			2E	*1E*
0.5	–	0.068		
1.0	–	0.120		
–	0.5	0.075		
–	1.0	0.126		
0.5	0.5	0.142	0.143	0.095
1.0	1.0	0.224	0.246	0.115

$GnGnF^3$-peptide, the third possible acceptor, could not be purified to complete homogeneity in terms of fucosylation. GnGn-peptide (usually 1–2%) always remained in the preparation. Traces of fucose were transferred into this glycopeptide mixture and were interpreted to be due to the GnGn-peptide impurity (data not shown).

Competition experiments

To determine whether the α1,3- and α1,6-fucosyltransferase activities are due to a single enzyme or to two distinct fucosyltransferases, competition experiments were carried out using GnGn-peptide and $GnGnF^6$-peptide as acceptors (Table 1). Although $GnGnF^6$-peptide can only be an acceptor for the α1,3-fucosyltransferase activity and GnGn-peptide is an acceptor for both activities, the agreement between the experimentally measured activities and the calculated values for two distinct enzymes demonstrates that the α1,3- and the α1,6-fucosylation is catalysed by two different enzymes.

Order of fucose transfer

At the moment MB 0503 cell extract is the only available source in which both α1,3- and α1,6-fucosyltransferase activities are readily detectable [4] and a remarkable amount of difucosylated structures occurs *in vivo* [5]. The aim of this work was to investigate the order of fucosylation steps in MB 0503 cell extract resulting in difucosylated products. For comparison, the Fuc-T C6 from porcine brain and Sf-9

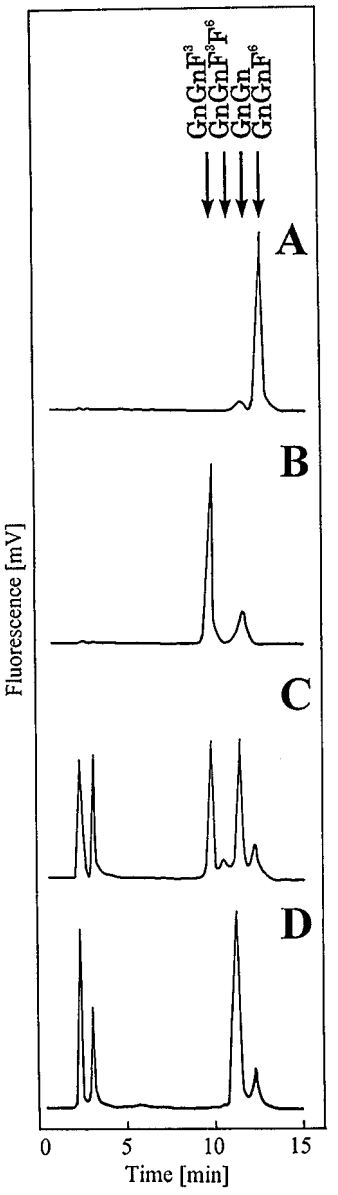

Figure 3. Incubation of GnGn-dNS with (A) Fuc-T C6, (B) Fuc-T C3, (C) MB 0503 cell extract and (D) Sf-9 cell extract. Elution positions of the standards are indicated by arrows. Incubation and HPLC conditions are described in the Materials and methods section. All chromatograms from incubations with insect cells contain early eluting peaks. These peaks are endogenous in the extract, since they occur even in the absence of acceptor.

cell extract, and the Fuc-T C3 from mung beans were tested under the same conditions.

Determination of glycosyltransferases by their ability to transfer radioactive sugars into acceptors and measuring incorporated radioactivity gives total enzyme activity, but transfer of the same sugar into different linkages cannot be detected. Previous work from this laboratory on the determination of the activity of glycosylation processing enzymes allowed a more detailed investigation of the linkage. Enzyme product was previously analysed using either pyridylaminated glycans directly, or glycopeptides digested with peptide:N-glycanase A and then pyridylaminated, followed by two dimensional HPLC [4, 15, 23, 24]. This method is not suitable in the present study because (i) fucosyltransferases transferring fucose to the inner GlcNAc do not act on pyridylaminated substrates [11] and (ii) no homogeneous $GnGnF^3$-glycopeptide was available.

For these reasons this work was carried out using dansylated glycopeptides. They have previously been shown to serve as suitable acceptors for core-fucosyltransferases and all required acceptors can be prepared to complete homogeneity [21]. The identification of the products was carried out by comparing their retention times with those of standards obtained from earlier studies [21] and by examining the results of digestion of the products with different amounts of α-L-fucosidase from bovine kidney. This enzyme removes the α1,6-linked core-fucose about 20 times faster than the α1,3-linked residue [15].

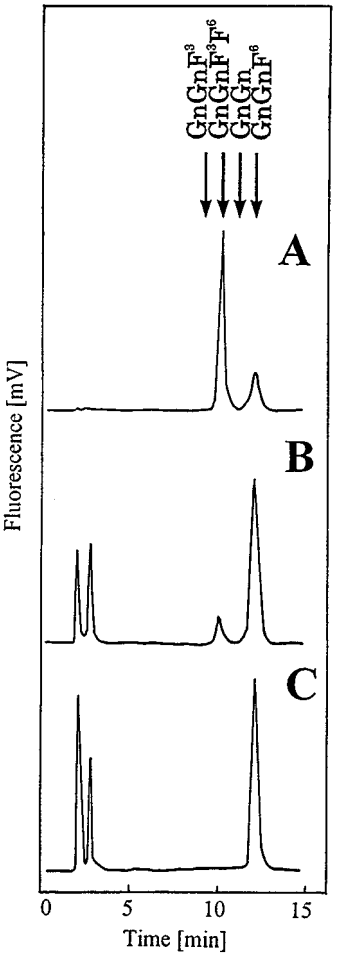

Figure 4. Incubation of $GnGnF^6$-dNS with (A) Fuc-T C3, (B) MB 0503 cell extract and (C) Sf-9 cell extract. Elution positions of the standards are indicated by arrows. Conditions are described in the Material and methods section.

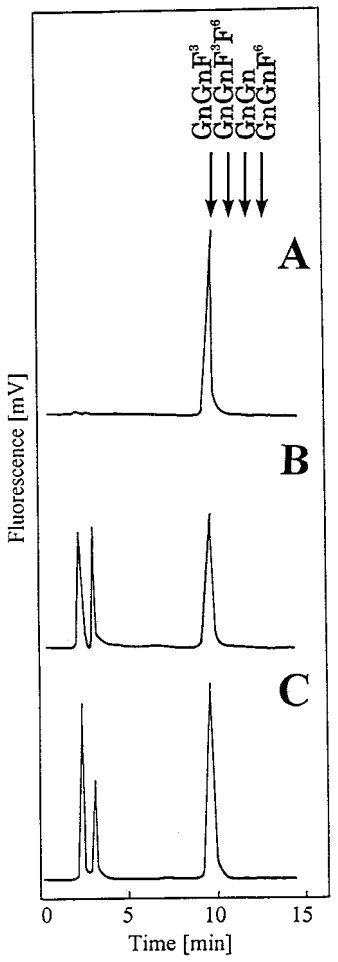

Figure 5. Incubation of GnGnF^3-dNS with (A) Fuc-T C6, (B) MB 0503 cell extract and (C) Sf-9 cell extract. Elution positions of the standards are indicated by arrows. Conditions are described in the Material and methods section.

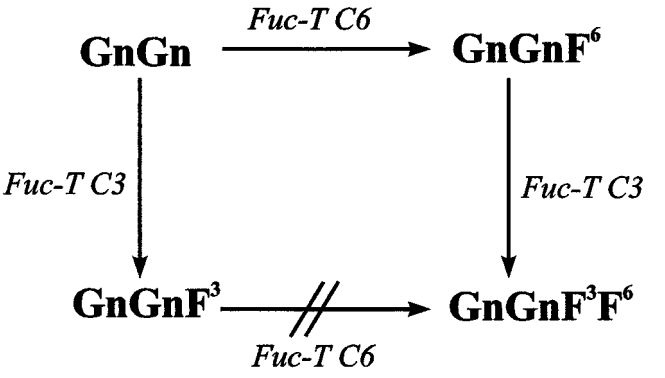

Figure 6. Biosynthesis of difucosylated structures.

(i) Incubation of GnGn-dNS

Porcine brain and mung bean fucosyltransferases gave, as expected, α1,6- and α1,3-monofucosylated products respectively when incubated with GnGn-dNS (Figure 3). After incubation with MB 0503 cell extract all three possible fucosylated structures (GnGnF^3, GnGnF^6 and GnGnF^{3}F^6) appeared, although only small amounts of the difucosylated structure were formed. Incubation with Sf- ∴ 9-extract gave only the GnGnF^6-dNS product. This result was not surprising since, although Sf-9 glycans also contain traces of α1,3-fucosylated structures [5], in previous studies the Fuc-T C3 activity was also too low to be detected [4].

Biantennary glycans or glycopeptides with a terminal GlcNAc residue linked β1,2- to the α1,3 antenna were shown to be excellent acceptors for fucosyltransferases [4, 7–9, 20]. The data shown here completely correlate with those previously seen for the glycopeptide acceptors and therefore provide additional proof that the dansylated glycopeptides are suitable substrates for both fucosyltransferase activities.

(ii) Incubation of GnGnF^6-dNS

Using GnGnF^6-dNS as an acceptor for the Fuc-T C3 from mung beans and the MB 0503 cell extract, difucosylated products were detected (Figure 4). These observations concur with those from earlier experiments, where the incubation of GnGnF^6-glycopeptides with the Fuc-T C3 from mung beans, MB 0503 cells or bee venom gland extract resulted in the formation of difucosylated structures [4, 11]. No difucosylation was observed after incubation with Sf-9 cells.

(iii) Incubation with GnGnF^3-dNS

Here, for the first time, a homogeneous α1,3-fucosylated substrate was incubated with Fuc-T C6 from various sources. Neither incubation of GnGnF^3-dNS with the Fuc-T C6 from porcine brain nor with one of the insect cell extracts resulted in a difucosylated product (Figure 5).

These results lead to the conclusion that the difucosylation of the inner GlcNAc of N-glycans – so far only known for insect cells – follows a strict order where α1,6-fucosylation occurs before α1,3-fucosylation. The α1,3-fucosylation of the inner GlcNAc seems to be a 'NO GO' signal for α1,6-fucosyltransferases (Figure 6).

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