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Review

The Glycosylation Capacity of Insect Cells

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It is generally accepted that insects primarily synthesise oligomannosidic and paucimannosidic *N*-glycan structures. Indeed, insects' capability to produce human-like complex type *N*-glycans has been a matter of controversy for a number of years. The relative or complete lack of these structures was primarily attributed to low (or undetectable) activities of the glycosyltransferases needed to drive the synthesis of hybrid and complex type *N*-glycans (*i.e.*, β-1,2-*N*-acetylglucosaminyltransferases I and II, β-1,4-galactosyltransferase, α-2,3- and α-2,6-sialyltransferases). Recent developments, fuelled by availability of genomic sequences and by advances in relevant methodologies, have shed some light on the subject, with a few unexpected twists. The identification of a transmembrane/Golgi hexosaminidase, an enzyme which removes a non-reducing *N*-acetylglucosamine residue during *N*-glycan biosynthesis, has demonstrated that the synthesis of complex-type *N*-glycans is actively and deliberately being prevented in insects. On the other hand, the characterisation of an active α-2,6-sialyltransferase in *Drosophila*, combined with the occurrence of sialylated *N*-glycan structures as detected in a detailed analysis of *Drosophila* embryos, has clearly shown that insects can, and need to, synthesise low levels of these structures. The current understanding of the insect *N*-glycan biosynthetic pathways taking place in Golgi apparatus and *trans*-Golgi network are elaborated and discussed.

Keywords
insect
N-glycan
sialic acid
glycoprotein
hexosaminidase

INTRODUCTION

The glycosylation of proteins, whether *N*- or *O*-linked, is a major category of post-translational modification in both eukaryotic and prokaryotic species; however, it is highly diverse – ranging from the *S*-layer glycoproteins of bacteria to the complex *N*-glycans present in vertebrates.[1] In recent years, the use of molecular biology has facilitated the decoding of numerous genes, including hundreds involved in glycosylation and has fuelled a need for adequate systems to enable the study of the encoded proteins. The amounts of protein from natural sources can be a significant hurdle for biological and medical applications; thus, the production of recombinant proteins has become a major goal with both academic and industrial significance. Since post-translational modifications affect protein function, the glycosylation of recombinant proteins is one factor which must be considered in their production.

THE FIRST STUDIES

Although insect cells are frequently used as hosts for the expression of recombinant proteins because of their eukaryotic post-translational modification capacities and ability to express larger quantities of recombinant protein, there is only limited data available on the naturally-occurring *N*-glycan structures in insects (see review, Ref. 2);

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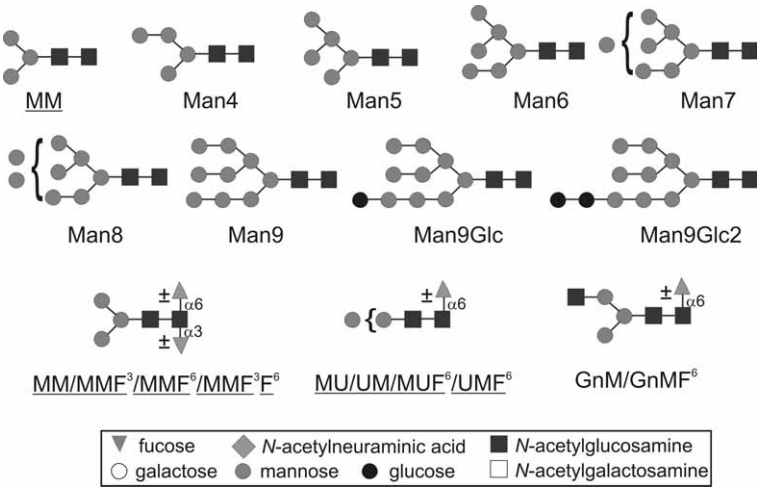

Figure 1. Structures commonly found in various insect species. The paucimannosidic structures are underlined. The glycans are depicted following the glycan nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org).

the presence of paucimannosidic *N*-glycans is seen as hallmark of insect glycosylation (for typical insect structures see Figure 1). One of the first studies examining endogenous insect *N*-glycans dealt with mosquito cells (*Aedes albopictus*) and has included pulse label experiments with ^{3}H-mannose followed by endo-β-*N*-acetylglucosaminidase H and α-mannosidase treatment of glycopeptides; the mosquito cells were found to contain high mannose and MM structures, but no complex oligosaccharides, were detected.[3] The existence of mannosidase-resistant labelled structures in these experiments could not be explained, although considering current understanding of insect *N*-glycosylation potential, these effects were potentially caused by the presence of paucimannosidic glycans, as well as the conversion of mannose to fucose, which is theoretically possible since GDP-fucose, the fucosyltransferase donor is derived from GDP-mannose.[4] Another of the initial articles on an insect glycosylation profile utilised another mosquito larval cell line (*Aedes aegypti*). Through a combination of sugar compositional analysis and enzyme digests (mainly α-mannosidases and endo-β-*N*-acetylglucosaminidase H from *Streptomyces griseus*), $Man_9GlcNAc_2$ was found as the major *N*-glycan structure on a membrane protein, whereas the presence of fucose (0.65 residues per GlcNAc residue) was neglected in the carbohydrate analysis.[5] Analysis of a haemolymph protein from *Manduca sexta* also only resolved $Man_9GlcNAc_2$ (*i.e.*, Man9).[6]

CORE FUCOSYLATION OF *N*-GLYCANS

The long-held belief that insects have the capacity to synthesise only high mannosidic structures was partly contradicted by sugar composition and permethylation analysis of the important honeybee venom allergen phospholipase A_2, on which the presence of non-reducing terminal GlcNAc and α-1,6-linked fucose was demonstrated, an indication for the insect's capacity to perform the first biosynthetic steps towards complex glycans.[7] Furthermore, in honeybee venom gland extract, a novel α-1,3-fucosyltransferase with the ability to convert GnGnF^6 into a difucosylated structure with two Fuc residues at the Asn bound GlcNAc was detected. The presence of more than one Fuc residue linked to the same monosaccharide on this structure was verified by NMR analysis. Since no transfer of fucose to either MM or MMF^6 was observed,[8] it became obvious that the prior action of β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) is necessary in insects, a result now confirmed with a recombinant form of the honeybee core α-1,3-fucosyltransferase.[9] The natural occurrence of the Fucα1,6(Fucα1,3)GlcNAc moiety on the PNGase A released paucimannosidic structures from bee-derived glycoprotein phospholipase A_2[10] as well as from hyaluronidase[11] corroborated the data on the α-1,3-fucosyltransferases, further demonstrating the existence of double-fucosylated *N*-glycan structures in insects (Figure 2).

As regards the fucosyltransferase activities, of the lepidopteran cell extracts from the MB-0503 (*Mamestra brassicae*), Bm-N (*Bombyx mori*) and Sf-9 (*Spodoptera frugiperda*) cells, only MB-0503 detectably converted the IgG GnGnF^6 glycopeptide into the difucosylated struc-

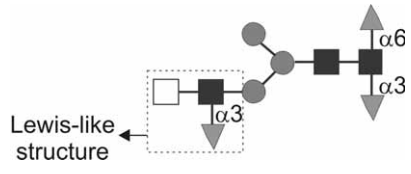

Figure 2. The most complex glycan structure found on bee venom glycoproteins. Apart from the difucosylated proximal GlcNAc residue, this structure carries a third fucose residue linked to the terminal LacdiNAc structure on the α-1,3-arm, thereby forming a Lewis-like structure. The glycan structure, later referred to as MGNFF^{3}F^6, is depicted using the same system as in Figure 1.

ture.[12] Also, analysis of PA-labelled glycans released with PNGase A from membrane glycoproteins of the three cell lines (Sf-21, MB-0503, Bm-N) was performed by 2D-HPLC in combination with exoglycosidase digests. MMF^6, MMF^3, MUF^6, MUF^3F^6, $MGnF^6$, $MGnF^3F^6$, $GnMF^6$ and $GnMF^3F^6$, but no MUF^3, $MGnF^3$ or $GnMF^3$, were found,[13] which is probably a result of relatively higher activity levels of the α-1,6-fucosyltransferase in the relevant Golgi compartment. Consistent with the detected activity, the MB-0503 cell line exhibited the highest degree of α-1,3-fucosylated glycans; the two other cell lines were also capable of synthesising these structures, albeit at low levels.[13] A further factor in determining the core fucosylation pattern is that α-1,6-fucosylation cannot take place after α-1,3-fucosylation.[14]

The presence of core α-1,3-fucose in Lepidopteran cell lines is not just of significance for what it says about the glycomic potential of insects in general, but is also of interest due to the use of these cells in biotechnology, since core α-1,3-fucosylation is known to be immunogenic and constitutes an epitope for antisera raised against plant glycoproteins such as horseradish peroxidase[15] as well as for IgE from patients allergic to plant and insect materials.[16] Thus, when considering hosts for production of proteins for potential therapeutic use, the presence of core α-1,3-fucose on specific recombinant glycoproteins must be examined. Indeed, a large amount of data as to the glycosylation potential of different insect cell lines has been gained by expressing various, mainly human, recombinant proteins (see Table I), with *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High Five) representing the most commonly-used insect cell lines; interestingly both these lines are ovary-derived and so their glycans do not necessarily reflect the complete glycomic potential of insect species. Certainly, the use of insect cells as expression systems is regarded as an option to circumvent the limitation of glycoprotein expression in microorganisms primarily due to the either complete lack of mammalian-type glycosylation when expressed in *E. coli* or the limitation to oligomannosidic structures (with potential hypermannosylation) when expressed in *Pichia pastoris* or other yeasts. Whereas the *Trichoplusia ni* cell line does add α-1,3-fucose to recombinant proteins,[17–20] this residue has not be detected on glycoproteins expressed in *Spodoptera frugiperda* Sf9 cells, even when the authors specially used PNGase A,[21] an enzyme known to release this type of glycans.[22] In most of the studies on recombinant glycoproteins from *Spodoptera frugiperda* cells, PNGase F or hydrazine have been used to liberate the oligosaccharides from the peptide chain. Both methods are known to result in a loss of information: the PNGase F is known not to release *N*-glycan structures containing α-1,3-fucose, whereas, in the case of hydrazinolysis process, the α-1,3-fucose substituent might be eliminated or result in artefacts.[23] Nevertheless, in a study published in 1991, hydrazine was applied to release oligosaccharides from *Drosophila melanogaster* glycoproteins. The expected well-known oligomannose series, as well as α-1,6-fucose linked to the inner GlcNAc on MM and MU, was found, whereas some structures could not be clearly identified due to their low occurrence. Interestingly, despite the carbohydrate-based immunological cross-reactivity between *Drosophila* neural tissue and horseradish peroxidase, when using anti-horseradish peroxidase (anti-HRP),[24-26] neither core α-1,3-fucose nor β-1,2-xylose (two substitutions typical for plants) were reported in this[27] or other[28] early studies which used hydrazine to release *N*-glycans from flies.

It was only in 2001, that the final piece in the puzzle about the basis for anti-HRP staining in *Drosophila* was found; then, finally, the presence of MMF^3F^6, as 1 % of the total *N*-glycan pool, could be proven in PNGase A released *N*-glycans from *Drosophila*. Also the relevant activity of a recombinant α-1,3-fucosyltransferase (FucTA) tested *in vitro* to transfer fucose in α-1,3-linkage to the innermost GlcNAc of $GnGnF^6$, GnGn, $GalGalF^6$ and GalGal, but not to MMF^6 nor to MM, was defined.[29] Furthermore, RNAi targeting the transcripts of FucTA was found to result in a decrease in anti-HRP reactivity of a *Drosophila* neural cell line.[30] Also, an endogenous C-type receptor binding to glycans with core α-1,3-fucose was found in the model organism,[31] whereas one specific male sex peptide was found to carry a difucosylated *N*-glycan.[32]

COMPLEX TYPE *N*-GLYCANS IN INSECTS

Amongs other *N*-glycan structures found on the bee venom phospholipase A_2, a structure containing a GalNAcβ1-4(Fucα1-3)GlcNAcβ1-2 moiety linked to the α-1,3-arm was detected (Figure 2). As previously mentioned, the identification of this structure was the first indication that insects are capable of performing chain-elongation in the direction of complex glycans starting with the transfer of GlcNAc to the α-1,3-arm.[33] Reappraisal of the bee venom glycans with FAB-MS[34] and, most recently, MALDI TOF-TOF MS[35] supported the findings published by Kubelka *et al.*, as has the recent definition of a recombinant honeybee enzyme capable of synthesising a fucosylated form of LacdiNAc.[9] However, the remaining *N*-glycans of the hymenopteran insect (bee) resemble those from lepidopteran (butterflies, moths) and dipteran (flies) species carrying mainly high-mannosidic and paucimannosidic structures.

In contrast, the orthoptera *Locusta migratoria* (locust) was found to produce unusual *N*-glycans with phosphorylethanolamine (AEP; aminoethylphosphonate) linked to the 6-position of Man or non-reducing terminal GlcNAc residues[36] (Figure 3a); the AEP moiety is also found on insect glycolipids[37] and wasp *O*-glycans.[38] On the other

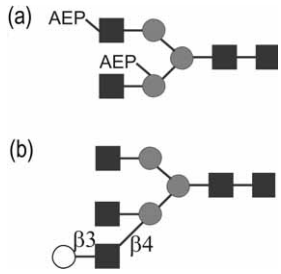

Figure 3. Unusual *N*-glycan features found on locust (a) and royal jelly glycoproteins (b). The glycan structures are depicted using the same system as in Figure 1; AEP, aminoethylphosphonate.

hand, NMR data of royal jelly glycoproteins, in combination with exoglycosidase digestions of PA-labelled glycans analysed by 2D-HPLC, led to the claim of a Galβ1-3(GlcNAcβ1-2)GlcNAcβ1-4 moiety on the α-1,3-arm, which could suggest the action of *N*-acetylglucosaminyltransferase IV (GnTIV) in the honeybee[39] (Figure 3b). Interestingly, a similar modification but with a β-1,4-linked galactose residue has been found in *Drosophila*.[40,41]

Indeed, the matter of galactosylation and/or sialylation in insects has, as also discussed below, generated the most controversy. Recently, though, an active *Drosophila* sialyltransferase[42] as well as very low levels of sialylated *N*-glycans in *Drosophila* embryos have been described,[40] thus verifying old data suggesting the presence of sialic acids in insects.[43] Interestingly, although a β-1,4-galactosyltransferase homologue in *Drosophila* can only transfer GalNAc,[44,45] the sialic acid in fly *N*-glycans is attached via β-1,4-galactose; thus, the nature of the relevant *N*-glycan modifying galactosyltransferase is still to be resolved.

GOLGI HEXOSAMINIDASE

In contrast to the more divergent glycans present on endogenous insect glycoproteins, for most of the glycoproteins expressed in insect cells, either from lepidopteran or dipteran species, the oligosaccharide side chains have been reported to consist either of the high mannosidic type or of truncated trimannosyl *N*-glycans with fucose α1,6-linked to the inner GlcNAc (see Table I, also for a review Ref. 46). In general, *N*-glycosylation sites occupied in native mammalian proteins by complex glycans (for a biantennary complex mammalian structure see Figure 5) were, in their recombinant insect forms, replaced by fucosylated MM structures or by processed mannosidic structures.[47-49] In addition, no trace of GnGn, GnGnF^6, GnGnF^3 nor of GnGnF^{3}F^6 on membrane glycoproteins of the three cell lines (Sf-21, MB-0503, Bm-N) was reported, although low levels of variants with MGn or GnM as well as paucimannosidic fucosylated species were present. These phenomena were explained by the action of an endogenous hexosaminidase specific for the GlcNAc in β-1,2-linkage to the α-1,3-arm and the rather low activity of β-1,2-*N*-acetylglucosaminyltransferase II responsible for the GlcNAc transfer to the α-1,6-arm.[13]

The relevant membrane bound Golgi β-*N*-acetylglucosaminidase, that was initially identified in a Golgi preparation of the insect cells, exclusively removed the GlcNAc residue from the α-1,3-arm when using GnGn-, MGn- and GnGnF^6-PA as substrates. This reaction is, therefore, indeed counteracting the chain elongation catalysed by GnTI and, subsequently, the formation of LacdiNAc units as found in bee venom.[50] Another example comes from a study comparing the glycans present on influenza virus haemagglutinin expressed either in *Spodoptera frugiperda* (Sf9) or in *Estigmene acrea* (Ea) cells, which showed a relative abundance of MM : MGn : GnGn of 90 : 10 : 0 in Sf9 cells *versus* 12 : 72 : 16 in Ea cells. Since Sf9 cells exhibited a 2.5-fold higher GnTI level, the apparent lack of GlcNAc on the glycans of these cells is due to the high endogenous β-*N*-acetylglucosaminidase activity removing GlcNAc preferentially from MGn; in contrast, in the Ea cell lysate only a minor hexosaminidase activity was present.[51] Indeed, very recently the relevant enzyme from *Drosophila* has been identified, clearly demonstrating the deliberate action of a β-*N*-acetylglucosaminidase in processing of *N*-glycans.[52] A deletion in the relevant fused lobes (*fdl*) gene has a major impact on the *Drosophila N*-glycan profile consistent with its *in vitro* activity. On the other hand, two newly-described hexosaminidases from Sf9 cells do not, though, possess the same exact specificity for *N*-glycans.[53,54]

Regarding the final structures of glycans of insect cell-produced proteins, other potential locations for hexosaminidase action should be considered. Glycan chains of proteins produced and secreted into the medium are accessible to the exoglycosidases (mainly β-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase, but also sialidase) that are present in the medium.[55,56] Also, in the supernatant of the cells from *Spodoptera frugiperda* (fall army worm), *Bombyx mori* (silkworm), *Trichoplusia ni* (cabbage looper) and *Malacosoma disstria* (forest tent caterpillar) infected with baculoviruses, β-*N*-acetylglucosaminidase activity was found to rise dramatically in the first 30 h post-infection. The proposed explanation was that the cell lysis occurring during viral infection leads to the release of endogenous glycosidases. As another source for the measured glycosidase activities in the media, non-heat inactivated foetal bovine serum should be considered too. Indeed, in an unused culture medium supplemented with 10 % foetal bovine serum, a weak endogenous activity of β-*N*-acetylglucosaminidase and β-galactosidase was detected.[55]

Examining the glycosylation capacity of insects has also included testing cell extracts for transferase activities. In cultured lepidopteran cells active GnTI and GnTII were found when incubating extracts of the cell lines with MM-, Man5- and MGn-PA. Compared to the human en-

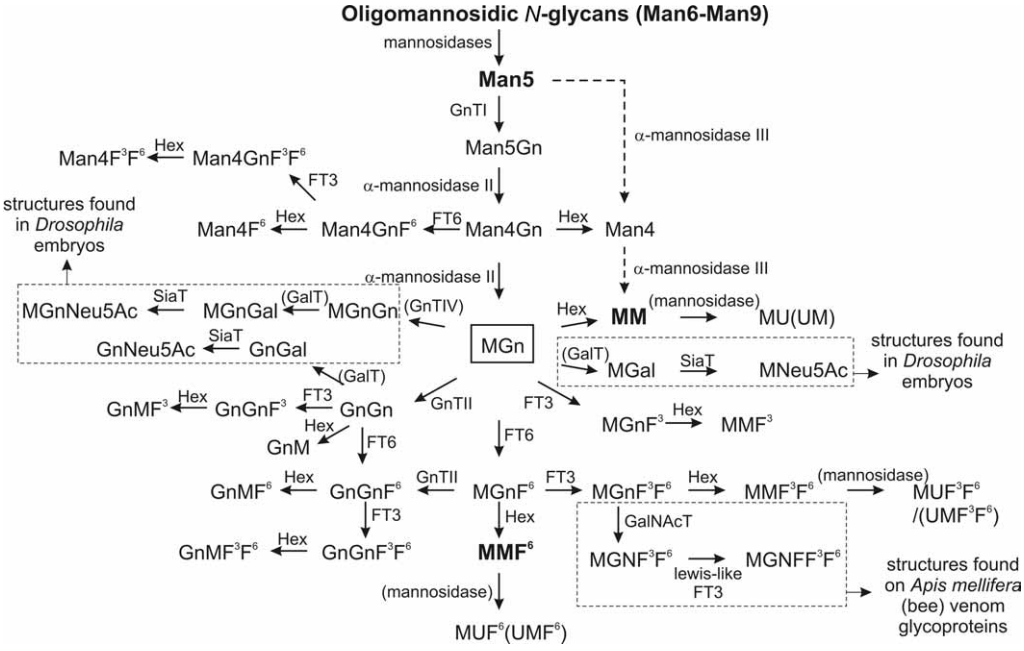

Figure 4. Current understanding of *N*-glycan biosynthetic pathways in the insect Golgi apparatus and trans-Golgi network. In insects, oligomannosidic *N*-glycans are processed by mannosidases as far as the Man5 structure, which serves as substrate for β-1,2-*N*-acetylglucosaminyltransferase I (GnTI). Man5Gn is either trimmed down by α-mannosidase II to MGn or is, via Man4Gn, substrate for either the Golgi β-*N*-acetylglucosaminidase (Hex) and/or the core α-1,6-fucosyltransferase (FT6), thus resulting in formation of Man4, Man4GnF^6 or Man4F^6. Through the action of core α-1,6-fucosyltransferase, Golgi β-*N*-acetylglucosaminidase and β-1,2-*N*-acetylglucosaminyltransferase II (GnTII), GnGn, GnM, GnGnF^6, GnMF^6, MM and MMF^6 are formed. MGnF^6, GnGnF^6 and Man4GnF^6 can be used as substrates by the core α-1,3-fucosyltransferase (FT3) leading to di-fucosylated structures. The transfer of the α-1,3-fucose precludes the subsequent action of the core α-1,6-fucosyltransferase, thus leading to GnGnF^3, GnMF^3, MMF^3, MGnF^3. A mannosidase, which seems to prefer α-1,3-arm mannose, is involved in creation of MU, MUF^{3}F^6 and MUF^6 structures. The dominant structures found in most insect species (Man5-Man9, MMF^6 and MM) are printed in bold. The enzymes whose activities are not clearly assigned to a specific protein/DNA sequence are in brackets. The MGNFF^{3}F^6 structure is depicted in Figure 2; the MGnGal and MGnGn structures are triantennary *N*-glycans with non-reducing terminal mannose on the α-1,6-arm and β-1,2- and β-1,4-linked GlcNAc residues on α-1,3-arm with or without a terminal galactose residue and are precursors of the sialylated structures shown in Figure 5b. The MGn structure (highlighted with a solid square) is a substrate for a number of enzymes: Golgi β-*N*-acetylglucosaminidase, FT3, FT6, β-1,4-galactosyltransferase (GalT), GnTII and β-1,4-*N*-acetylglucosaminyltransferase IV (GnTIV); the result of this competition determines the final, relative amounts of complex (*e.g.*, GnGn), hybrid (*e.g.*, MGn) and paucimannosidic structures (*e.g.*, MMF^6). The structures highlighted with dotted squares were found only in *Drosophila* embryos/on bee venom glycoproteins. *In vitro* analysis of Sf9 α-mannosidase III suggested that a direct route from Man5 to MM (and possibly MU and UM) may exist.[57]

zymes, the transfer rate of the insect GnTI to MM-PA was 10 times less effective while the physiologically relevant Man5-PA was equally processed. On the other hand, the amount of GnTII activity (at least when tested with MGn-PA; the potentially relevant MGnF^6-PA was not tested) was some 72-400 times less than in a mammalian cell line. When testing the fucosylation rate of various glycans, MGn-peptide was processed at half the rate compared to GnGn-peptide while MM-peptide was not a substrate. Thus, the α-1,3-arm GlcNAc constitutes the »go« signal for the α-1,6-Fuc-T. This GlcNAc residue is not present on the paucimannosidic fucosylated structures and therefore has been proposed to have a transient role in the biosynthesis of insect glycans[58] (Figure 4). Also, a β-1,4-*N*-acetylgalactosaminyltransferase was found to be expressed in lepidopteran cell lines capable of transferring GalNAc from UDP-GalNAc in β-1,4-linkage to terminal β-linked GlcNAc, thereby, synthesizing the complex type LacdiNAc unit as found in the bee venom, whereas only a minor β-4-galactosyltransferase activity was detected in these cells.[56] Relevant β-1,4-*N*-acetylgalactosaminyltransferases from *Trichoplusia ni* and *Drosophila* have been expressed in a recombinant form;[59,60] the *Drosophila* β-1,2-*N*-acetylglucosaminyltransferase I has also been characterised[61] and shown to play an important role in normal *N*-glycan biosynthesis in the fly.[62]

SIALYLATION IN INSECTS

A common feature on complex type *N*-glycans in mammals is terminal sialylation (Figure 5a). To understand the (near) absence of sialylated, complex glycans in insects the availability of the nucleotide sugars was studied. HPAEC of lysed cell extracts from Sf9 cells and High Five cells grown in serum free medium demonstrated the absence of CMP-NeuAc while the concentrations of UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc and GDP-Man were equal to, or higher than, those reported in CHO cells. In gene-

ral, High Five cells showed the highest concentrations of nucleotide sugars other than GDP-Man.[63]

With the exception of the recent data on *Drosophila* embryos, analyses of a variety of insect species by a number of groups failed to find complex-type glycans modified with sialyl-residues. Nevertheless, one group detected sialylated *N*-glycan structures on recombinantly expressed plasminogen in insect cells. These authors used a combination of exoglycosidase digestions and HPAEC; monosaccharide analysis was performed after digestion with a clam exoglycosidase mixture and separation of the released monosaccharides from the enzyme mixture with an ultrafiltration device. The elution profile of the monosaccharides and the oligosaccharides were compared with commercial standards. Their first published data on *Spodoptera frugiperda* SF21 AE cells showed mainly Man9, Man5, Man4 and MM and about 20 % of a sialylated biantennary structure when the plasminogen was collected for 48 h post-infection.[64] In another cell line (MB-0503) tested by the same authors even more complex structures like asialo biantennary (GalGal, 7 %), and fucosylated asialo biantennary (GalGalF^6, 3 %), bisialo biantennary (28 %) and fucosylated bisialo biantennary (25 %) were found.[65] The same lab handled at the same time 23 different insect cell lines from *Spodoptera frugiperda, Spodoptera exigua, Mamestra brassicae, Trichoplusia ni, Anticarsia gemmatalis, Choristoneura fumiferana, Estigmene acrea, Heliothis viresens, Heliothis zea, Lymantria dispar, Manduca sexta* and *Plutella xylostella* in six different media (depending on the used cell line).[66] In the same lab, Chinese hamster ovary (CHO) cells expressed plasminogen with bi-, tri- and tetraantennary complex type structures containing variable amounts of sialic acids in α-2,6 and α-2,3 linkage as well as MM, MMF^6, GnGnF^6, $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$.[67] The previously mentioned Sf21 AE cells were found to express recombinant plasminogen with α-2,6- and α-2,3-sialylated bi-, tri- and tetraantennary glycans when the protein was harvested 96 h post-infection; multiantennary glycans have, though, not been found by others studying glycans from this cell line. Furthermore, this group claimed that the amount of complex glycans correlates with the length of infection time.[68] Nevertheless, while other labs nowadays put a lot of effort to humanize insect cell lines, these »already« humanized lines were put aside to continue expression in *Pichia pastoris*[69] as well as revisiting insect cells with expression of plasminogen in *Drosophila* S2 (macrophage-like) cells, but without examining the glycan profile.[70]

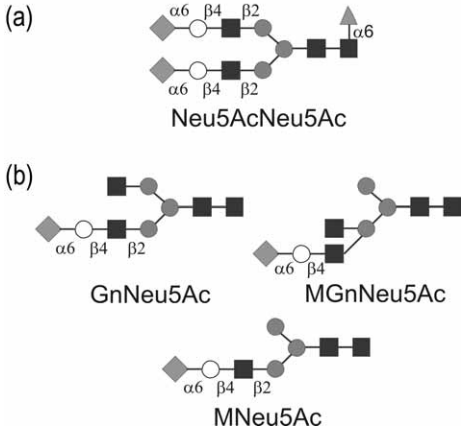

Figure 5. Relevant *N*-glycan structures with terminal sialic acid. Example of a typical mammalian structure with terminal sialic acid residues and α-1,6-fucose on the core (a). Sialylated structures found in *Drosophila* embryos lack the fucose residues and carry only one sialic-acid residue (b). Sialylated structures were until recently considered not to be present in wild-type insect cells; a detailed analysis of *N*-glycans from *Drosophila* embryo confirmed their presence, albeit in a very low amount.[40] In this study, the galactose residues were found to be (β-1,4)-linked to GlcNAc residues, unlike the (β-1,3)-linked residues found on royal jelly glycoproteins[39] (also see Figure 3). The glycan structures are depicted using the same system as in Figure 1.

In other labs, lectins such as *Sambucus nigra* agglutinin (SNA) have been used, in conjunction with sialidase treatment, to claim the presence of sialic acid on insect glycoproteins.[71,72] In our lab, however, SNA was found to also bind GalNAc-carrying *N*-glycans (unpublished data), a possible feature in insects since the relevant transferase activity has been demonstrated *in vitro*; interestingly, the sialidases used were tested for numerous glycosidase side-activities, but not, according to the manufacturer's information sheet, for GalNAcase activity. Although the relevant α-2,6-sialyltransferase activity could not be detected in Sf9 cell line extracts,[73] recently it was demonstrated that *Drosophila* does express an active α-2,6-sialyltransferase.[41] Furthermore, detailed analysis of *N*-glycans of *Drosophila* embryos revealed a minute amount of sialylated *N*-glycan structures[40] (also see Figure 5b).

It is clear that structural determination of oligosaccharides is tedious work; due to various methods and applied techniques employed, it is difficult for other researchers to compare results of varying precision or reliability. Analyses employing HPLC-mapping of fluorescent labelled glycans, together with exoglycosidase digests and mass-spectrometric methods, appear to reveal more of the occurring glycosylation pattern than other approaches. During the last decade glycan analysis was very much fine-tuned partly due to the use of advanced mass-spectrometric methods. The increased sensitivity of modern methods makes structures with relative low abundance accessible for analysis, but, at the same time, increases the need to avoid sample impurities which can then also be easily reflected in the results. For example, many of the proteins expressed in insect cells are mainly purified on immunoaffinity columns, from which a bleeding of the bound antibody cannot be excluded. Such material would result in co-analysis of the glycan structures present on the antibody used.[74]

A lot more caution has to be taken when the insect cells are grown in a culture supplemented with serum or

fetuin. If then sialic acid-containing tri- or tetraantennary structures are found, it is very likely that the analysed structures are derived from fetuin that could not be separated from the recombinant protein by the single step purification. Suspicion should also arise when sialyated or galactosylated biantennary, triantennary and tetraantennary structures are reported while the precursors are not present and when, at the same time, the cells used to produce the glycoprotein contain no detectable activity of the relevant transferases. Additionally, when studying the glycosylation potential of cells one should choose a reporter glycoprotein that is not already present in the uninfected cells and/or medium;[75-77] certainly, serum-free medium is to be preferred in such experiments. In other studies, the rather unusual effects of microgravity[76] or hexosaminidase inhibition[72] as regards the appearance of sialylated structures in insect cells have been reported, although the latter, in the light of recent identification of *N*-glycan processing β-*N*-acetylglucosaminidase, might be of interest.

ENGINEERING GLYCOSYLATION IN INSECT CELLS

From the previous results, it was in generally clear that GnTI, GnTII, β-1,4-galactosyltransferase and sialyltransferases were the crucial enzymes limiting the glycan structures in insect cells (for a review see Ref. 78). Human GnTI was the first enzyme which, when expressed in Sf9 cells, led to an increase in the amount of terminal GlcNAc residues.[79] Furthermore, expression of mammalian β-1,4-galactosyltransferase in Sf9 cells to extend the *N*-glycosylation pathway in insects resulted in a galactosylated end-product.[80] Similar results were obtained when expressing the β-1,4-galactosyltransferase in *Trichoplusia ni* cells. The galactose residues were attached exclusively to the α-1,3-arm, although a trace amount of GnM was also present. The action of the arm-specific hexosaminidase was then inhibited by galactosylation yielding MGal, $MGalF^6$ and $MGalF^3F^6$; obviously, the level of GnTII activity was not sufficient to compete for the same substrate as the recombinantly expressed β-1,4-galactosyltransferase, which itself has a bias towards the α-1,3-arm. Nevertheless, a significant reduction of MMF^3F^6, MMF^6, $MGnF^6$ and MUF^6 was observed, the latter perhaps being a product of an α-mannosidase III[54] or an unknown α-1,3-mannosidase.[81] Therefore, to humanise Sf9 cells further, they were additionally engineered with GnTII and sialyltransferases (Figure 6). When grown in medium supplemented with bovine fetuin these cells indeed were able to produce biantennary, terminally sialylated *N*-glycans.[82] These cells did not, however, synthesise sialylated *N*-glycans when cultured in serum free medium or when supplemented with asialofetuin. Since Sf9 cells have only low levels of sialic acid and no detectable CMP-sialic acid, evidence for a sialic acid salvage pathway was strengthened by data indicating that sialylation occurred when the cells were grown in medium supplemented with fetuin or terminally sialylated *N*-glycans.

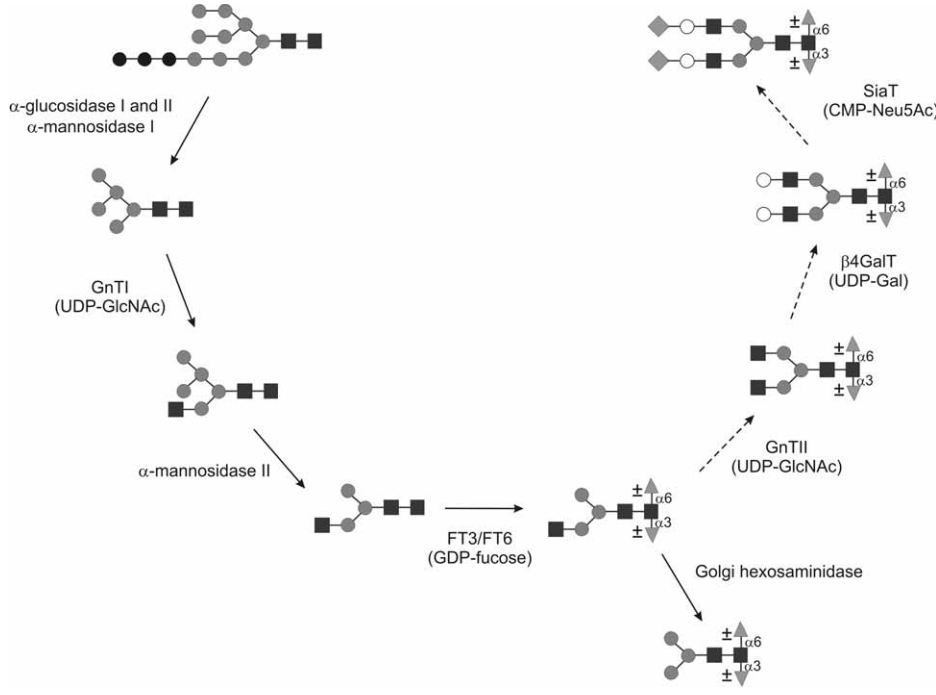

Figure 6. »Humanisation« of the *N*-glycan processing pathway in insect cells. The dotted arrows indicate pathways which have to be modified to induce increased levels of complex glycans.[85] The sugar-donor substrates needed for the respective reactions are in brackets. The glycan structures are depicted using the same system as in Figure 1.

TABLE I. Summary of insect cell lines used to express mammalian glycoproteins[a]

Insect cell line	Expressed protein; Polypeptide size (amino acids, aa, and / or MW of apo-protein); Harvesting time post infection (pi)	Used medium; Promoter	Number of potential *N*-sites	Glycan structure present	Used method	Ref.
Spodoptera frugiperda	Human erythropoietin; 193 aa, MW: 18400; 48 h pi	TNMFH + 10 % foetal bovine serum; Polyhedrin		Different glycosylation pattern to human	Size decrease on SDS-PAGE after PNGase F digest	87
Spodoptera frugiperda Sf9	Human tissue plasminogen activator; 562 aa, MW: 59000; 28 h pi	TNMFH + 10 % foetal bovine serum; Polyhedrin	2–3 out of 4	Possible structures: high-mannosidic glycans and MM	Partially Endo H resistant glycans, removed by PNGase F	88
Spodoptera frugiperda	Haemagglutinin of fowl plague virus; Precursor: 550 aa MW of HA1: 35200 MW of HA2: 25200	TC-100 + 10 % foetal bovine serum; Polyhedrin	HA1: N^{12} N^{28} $N^{123}N^{149}$ N^{231} HA2: $N^{406}N^{478}$	MM, MMF^6 $Man_{5\text{-}9}GlcNAc_2$	Size decrease on SDS-PAGE with Endo H, Endo D and Endo F radioactive labelled with 2-H^3 mannose, PNGase F released oligosaccharides, separated after exo-digest (A, B, C, D) with HPLC/Gel chromatography	47
Spodoptera frugiperda IPLB-F21AE	Human plasminogen; 791 aa; 48 h pi	Serum free; ExCell-400	N^{289}	$Man_{3\text{-}5}GlcNAc_2$ $Man_9GlcNAc_2$ 20 % disialo-biantennnary glycans	Lectin binding with CBL after neuraminidase treatment PNGase F released, analysis of oligosaccharides after neuraminidase digest and of monosaccharides after clam (*Venus mercenaria*) liver extract digest with HPAEC compared to standards from Dionex Comp.	64
Mamestra brassicae MBO 503	Human plasminogen; 791aa; 48 h pi	Serum free; ExCell-400	N^{289}	50 % disialo (α-2,6)-asialo-biantennnary glycans +/–F^6 MM, Man5, $Man_9GlcNAc_2$	Lectin binding with SNA, PNGase F released, analysis of oligosaccharides after sequential exo-digest (A, B, E-I) and of monosaccharides after clam (*Venus mercenaria*) liver extract digest with HPAEC compared to standards from Dionex Comp.	65

Spodoptera frugiperda Sf9	Chimeric respiratory Syncytial virus protein; MW of F: 59000 MW of G: 33000; 48 h pi	Grace's medium + 10 % foetal bovine serum	5–6 out of 8	MMF^6, mole ratio of fucose: 1.5	Radioactive labelled with 6-H^3 glucosamine, ConA and Pea lectin chromatography of glyco-peptides, hydrazinolysis, exo-digest (A, B), periodate oxidation for fucose linkage, monosaccharide analysis on Dionex	89
Spodoptera frugiperda Sf9	β subunit of human choriogonadotropin; 165 aa, MW: 18000; 96 h pi	Grace's medium +10 % foetal bovine serum, yeast hydro-lysate, lactalbumin; Polyhedrin	2	High-mannosidic, fucose content 2.5 *vs.* 1.8 in native form	Size decrease on SDS-PAGE with PNGase F, Endo H and Endo F, ConA binding, monosaccharide analysis on Dionex after TFA hydrolysis	90
Bombyx mori Silk worm larvae	Mouse interleukin-3; 160 aa, MW: 15700 from haemolymph		4 sites	$Man_2GlcNAc_2$ $Man_{2\text{-}4}$ (Fuc)$GlcNAc_2$	PNGase F released glycans, reductive amination (+n-hexylamine), and permethylation, LSI-MS-FAB	91
Bombyx mori Silk worm larvae	Mouse interleukin 3; 140 aa, MW: 15700 from haemolymph		2 out of 4 $N^{16}N^{86}$	$Man_{2\text{-}4}$ (Fuc)$GlcNAc_2$ $Man_2GlcNAc_2$	PNGase F released glycans, LSI-MS on tryptic peptides before and after deglycosylation to define *N*-sites	92
Spodoptera frugiperda Sf21	Human interleukin 2; 144 aa, 25% secreted; 4/7 d pi	TC-100 + 10 % foetal bovine serum; Polyhedrin	1 artificial	$Man_{2\text{-}3}$ (Fucα-1,6)$GlcNAc_2$	PNGase F released, methylation for composition on GC, permethylated oligosaccharides on FAB-MS	93
Spodoptera frugiperda Sf9	envelope protein Gp120 of HIV-1 BH8; 479 aa		22 sites	$Man_{5\text{-}9}GlcNAc_2$	Monosaccharide analysis after TFA on Dionex, PNGase F released glycans on HPLC; ^{1}H-NMR	48
Spodoptera frugiperda Sf9	Human interferon ω1; 164 aa; 4/6 d pi	Polyhedrin	N^{78}	MMF^6, MOF^6, no α-1,3-Fuc found	MS of tryptic glycopeptides PNGase A released glycans, HPLC after pyridylamination	22
Spodoptera frugiperda Sf9	Porcine interferon γ; 144 aa, MW: 17000; 64 h pi		2	High-mannosidic and hybride glycans	Binding with GNA, SNA, MAA, ConA of Dig glycan differentiation kit, no binding to SNA and MAA after sialidase treatment_	71

(cont.)

Spodoptera frugiperda Sf9	Soluble human interferon γ receptor; 226 aa, MW: 26000; 4–5 d pi	ILP/41, 0.4 % yeastolate, 1.5 % foetal bovine serum, cod oil	4 out of 5 N^{17} N^{62} N^{69} N^{162}	MMF^6, Man4, $MGnF^6$, M'MGn	PNGase F released glycans on MALDI-TOF, monosaccharide composition compared to standards on Dionex	94
Spodoptera frugiperda Sf9	Human interferon γ; 166 aa; 48 h / 96 h pi	TC 100 + 10 % heat inactivated foetal bovine serum; Polyhedrin	N^{25} N^{97}	$Man_{5\text{-}8}GlcNAc_2$ MM, MO, MGn MMF^6, $OGnF^6$ No α-2,6-sialyltransferase activity found	Mass difference on MALDI-TOF of glycosylated and PNGase F deglycosylated peptides confirmed with exo-digest	73
Estigma acrea Ea4	Human interferon γ; 166 aa; 48 h / 96 h pi	TC 100 +10 % heat inactivated foetal bovine serum; Polyhedrin	N^{25} N^{97}	$Man_{5\text{-}8}GlcNAc_2$, MM, MGn, MOF^6, MMF^6, $MGnF^6$, GnGn, $GnGnF^6$, GalGn, $GalGnF^6$, $GalGnF^6$ bisect, No α-2,6-sialyltransferase activity found	Mass difference on MALDI-TOF of glycosylated and PNGase F deglycosylated peptides confirmed with exo-digest	73
Mamestra brassicae	Bovine lactoferrin; 708 aa, MW: 73000; 5 d pi	Serum free medium; Polyhedrin	N^{233} N^{476} N^{545}	$Man_{5\text{-}9}GlcNAc_2$ MM, MMF^6, MGn, MO MOF^6, M'MGn	Size decrease on SDS-PAGE with PNGase F, monosaccaride composition with GC/MS (no sialic acid residues); glycopeptides on ESI-MS and CID-MS-MS; NMR after hydrazinolysis	49
Spodoptera frugiperda Sf9	Human cyclooxygenase 2; 603 aa; 65–73 h pi	Grace´s medium + 10 % foetal bovine serum	N^{53} N^{130} N^{396} N^{580} out of 5	High mannosidic glycans	Size decrease on SDS-PAGE with Endo H; FACE glycan profiling: PNGase F released glycans with fluorescent label on PAGE	95
Trichoplusia ni (TN-5B1-4) High Five	IgG; 8 % acidic glycans; 72 h pi	ExCell 401 or ExCell 405; Polyhedrin		On intracellular forms: 56 % $Man_{5\text{-}9}GlcNAc_2$ 35.6 % truncated $MGnF^6$, MMF^6, MGn, $M'MOF^3F^6$ 10 % complex: GnGal, $GnGnF^6$ On secreted forms: 5 % $Man_{5\text{-}9}GlcNAc_2$ 63 % truncated $MGnF^6$, $GnMF^6$, MMF^6, MGn, $M'MOF^3F^6$ 33 % complex: GnGal, $GnGnF^6$, $GalGnF^6$	PNGase A released glycans, 2D-HPLC of PA-labelled glycans, exo-digest (A, B, F, K), NMR, ESI-MS	17

Lymantria dispar, Heliothis virescens, Bombyx mori Larvae: *Spodoptera frugiperda, Trichoplusia ni, Heliothis virescens, Bombyx mori, Danaus plexippus*	Human placental secreted alkaline phosphatase (SEAP); 510 aa; 4–7 d pi	Cell lines: TNM-FH + 10 % foetal bovine serum; Larvae: Wheat germ diet or leaf based diet	≈2 sites	$Man_{4\text{-}7}GlcNAc_2$ MM, MMF^6, $Man4F^6$, MOF^6, MO, MM' In all expression systems 50 % fucosylated structures, except *B. mori* larvae (17 %)	FACE glycan profiling: PNGase F released glycans with fluorescent label on PAGE; exo-digest (A, B)	96
Trichoplusia ni	Murine neuropsin; 250 aa		N^{95}	$Man_{5\text{-}9}GlcNAc_2$ MM, MMF^6, MMF^3F^6 $GnMF^6$, MGn, MOF^6, MOF^3F^6, OOF^3F^6	PNGase A released glycans, 2D-HPLC of PA-labelled glycans, exo-digest (A, B), NMR	18
Trichoplusia ni High Five	Third eight cysteine domaine of the latent TGF-ß-binding protein; 80 aa; 60 h pi	ExCell-401 serum free; Polyhedrin	1 site	MM, MMF^6, $(M)OF^6$, $M(Gn)F^6$, $GnGnF^6$, MMF^3, $M(Gn)F^3$, $GnGnF^3$, $(M)OF^3$ MMF^3F^6, $GnGnF^3F^6$, $M(Gn)F^3F^6$, $(M)OF^3F^6$, GnGn, M(Gn), M(Gal), $O(Gal)F^6$, $M(Gal)F^3F^6$	Hydrazine released glycans on MALDI, PNGase F resistant, fluorescent labelling with 2-AB, HPLC, combined with exo-digest (B, G, H, L)	19
Spodoptera frugiperda Sf9	Third eight cysteine domaine of the latent TGF-ß-binding protein; 80 aa; 60 h pi	ExCell-401 serum free; Polyhedrin	1 site	MM, MMF^6, $M(Gn)F^6$, $GnGnF^6$	Hydrazine released glycans on MALDI-TOF, PNGase F sensitive; fluorescent labelling with 2-AB for HPLC combined with exo-digest (B, G, H, L)	19
Spodoptera frugiperda Sf9	Aspartic protease (Asp-2); 500 aa		N^{153} N^{172} N^{223} N^{354}	$Man_{5\text{-}8}GlcNAc_2$ MMF^6, MM, $MGnF^6$	PAGE separation, bands alkylated reduced and PNGase F treated, fluorescent labelled with AA-Ac, analysed on HPLC and MALDI-TOF	97
Trichoplusia ni High Five	Human interleukin-3; 133 aa; 72 h pi	ExCell-401	N^{15} N^{70}	MMF^3F^6, MMF^6 OMF^3F^6, OMF^6	PAGE separation, bands alkylated, reduced and trypsin treated, analysed on HPLC-ESI	20

(cont.)

Lymantria dispar 652Y	Human serum transferrin; 700 aa; 120 h pi	ExCell-420 +/– 10 % foetal bovine serum; Polyhedrin	2 sites	$Man_{5\text{-}8}GlcNAc_2$ OO, MO, MOF^6, MM, MMF^6, GnM $GnMF^6$ high GnTII level found	PNGase A of glycopeptides 2-PA labelled glycans on 2-D HPLC compared to standard glycans exo-digest (F, B, K) MALDI-TOF	98
Spodoptera frugiperda Sf21 *Trichoplusia ni* High Five (TN-5B1-4) Tn-4s	Human colorectal cancer A33 antigen; 300 aa; 3–4 d pi	ExCell-400, ExCell-405; TNMFH + 10 % foetal bovine serum	3 sites	$Man_{5\text{-}9}GlcNAc_2$ MM, MMF^6, $M(Gn)F^6$, GnGn	PNGase A or F released glycans from heat denatured protein 2-AB labelled, analysed on HPLC after exo-digest (A, B, F, M, N)	74
Drosophila Schneider S2	Urokinase-type plasminogen activator receptor; 283 aa, MW: 35000;	*Drosophila* SFM	N^{52} N^{162} N^{172} N^{200} out of 5	MMF^6	PNGase F digest, MALDI-TOF	99
Drosophila Schneider S2	Human erythropoietin; 165 aa, MW: 20000	M3 Shields and Sang medium	N^{24} N^{38} N^{83}	MMF^6, MM, MO, MOF^6	PNGase A digest of glycopeptides 2-PA labelled glycans on 2-D HPLC compared to standard glycans	100

[a] The *N*-glycans present were analysed with various methods as mentioned briefly in the table. In the cases were part of the structure is put in brackets, *e.g.* $M(Gn)F^6$ the substituted arm (α-1,3 or α-1,6) was not defined. M'MGn defines a Man4-type structure carrying a GlcNAc on the α-1,3-antenna and M'MO a linear structure of the form Manα1,3/6Manα1,6Manβ1,4GlcNAcβ1,4GlcNAc.

[b] TNMFH: Grace's medium supplemented with lactalbumin hydrosylate and yeastolate.

[c] Endo H treatment removes high mannosidic, Man5, but neither MM, MMF^6 nor MGn; resistance is thus no proof for complex glycans.

[d] PNGase F from *Flavobacterium meningosepticum* removes high mannosidic and complex mammalian *N*-glycans.

[e] A: α-1,2/3/6-mannosidase from jack bean, B: α-1,6-fucosidase from bovine kidney, C: α-1,2-mannosidase from *Aspergillus oryzae* or *phoenicis,* D: β-mannosidase from *Polyporus sulfureus,* E: β-1,2-mannosidase from *Turbo cornufus,* F: β-1,2/3/4/6-HexNAcase from jack bean, G: β-1,2-HexNAcase from *Diplococcus pneumoniae*, H: β-1,4-galactosidase from *Diplococcus pneumoniae,* I: α-2,6(3/8)neuraminidase from *Arthrobacter ureafaciens,* J: β-mannosidase from snail, K: β-1,4-galactosidase from jack bean, L: fucosidase from almond meal, M: β-galactosidase from bovine testis, N: sialidase from *Clostridium perfringens.*

This salvage pathway involves endocytosis of sialoglycoconjugates from the medium, desialylation by lysosomal sialidases and release of free sialic acids to the cytoplasm while in the Golgi CMP-sialic acid is used as donor.[83] Interestingly, a functional Golgi-targeted *Drosophila melanogaster* CMP-sialic acid synthetase has recently been found.[84] Furthermore, as mentioned above, a recent study using chymotrypsin and trypsin to release glycopeptides from *Drosophila* fly embryo powder discovered traces of monosialylated biantennary glycans thus proving the presence of sialic acid on a natural fly glycan.[40]

For future therapeutic applications, the reduced half-life of recombinant glycoproteins in the circulation due to altered glycosylation and the possible presentation, on these proteins, of immunogenic determinants such as α-1,3-fucose are important issues. Thus, efforts directed at engineering the glycosylation of these proteins by manipulating the processing enzymes in insect cells could include repressing the β-*N*-acetylglucosaminidase and α-1,3-fucosyltransferase and/or introduction of additional enzymes that would further »humanise« insect *N*-glycans. Such strategies have become more realistic due to the recent identification of insect hexosaminidases[52-54] and core fucosyltransferases.[9,29,86] Therefore, additional work is still required to identify relevant genes expressed in commonly-used cell lines and to re-engineer these to generate the 'ultimate' cell line with proper human-like glycosylation.

ABBREVIATIONS

AB, aminobenzene; PA, pyridylamino; HPAEC, High-Performance Anion-Exchange Chromatography; HPLC, High-Performance Liquid Chromatography; GnTI and GnTII, β-1,2-*N*-acetylglucosaminyltransferases I and II; GnTIV, β-1,4-*N*-acetylglucosaminyltransferase; FT3, α-1,3-fucosyltransferase; FT6, α-1,6-fucosyltransferase; GalNAcT, β-1,4-*N*-acetylgalactosaminyltransferase; SiaT, α-2,6-sialyltransferase; GalT, β-1,4-galactosyltransferase; Hex, Golgi β-*N*-acetylglucosaminidase; GalNAcase, *N*-acetylgalactosaminidase; LacdiNAc, GalNAcβ1-4GlcNAc; PNGase, Peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase; AEP, aminoethylphosphonate; SNA, *Sambucus nigra* agglutinin.

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SAŽETAK

N-glikozilacija u insekata

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Smatra se da insekti uglavnom sintetiziraju oligomanozni tip *N*-vezanih šećera. Sinteza *N*-vezanih šećera tipičnih za ljudski organizam u insekata više je godina bila tema rasprave. Niska razina ili potpuni nedostatak tih šećera pripisivana je niskoj (ili nemjerljivoj) razini aktivnosti glikoziltransferaza potrebnih za sintezu hibridnog i kompleksnog tipa *N*-vezanih šećera (konkretno: β-1,2-*N*-acetilglukozaminiltransferaze I i II, β-1,4-galaktoziltransferaza, α-2,3- i α-2,6-sijaliltransferaze). Istraživanja novijega datuma, potaknuta dostupnošću sekvencija genoma i napretkom u odgovarajućim metodološkim područjima, dala su novi uvid u to područje uključujući i neke neočekivane rezultate. Otkriće heksozaminidaze u Golgijevu kompleksu, enzima koji uklanja *N*-acetilglukozamin s nereducirajućeg kraja *N*-vezanih šećera tijekom njihove sinteze, pokazalo je da je sinteza ljudskog tipa *N*-vezanih šećera aktivno i namjerno potisnuta u insekata. S druge strane, otkriće aktivne α-2,6-sijaliltransferaze u vinskoj mušici, uz dokaz prisutnosti *N*-vezanih šećera modificiranih sijalinskom kiselinom u embrijima vinske mušice, jasno je pokazalo da insekti mogu i moraju sintetizirati male količine tih šećernih struktura. U ovom se članku obrazlaže i raspravlja trenutni stadij razumijevanja sinteze *N*-vezanih šećera u Golgijevu kompleksu i *trans*-Golgijevoj mreži insekata.