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Glycosylation in Lepidopteran insect cells: identification of a β1 → 4-*N*-acetylgalactosaminyltransferase involved in the synthesis of complex-type oligosaccharide chains

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The choice for a heterologous expression system to produce glycoprotein therapeutics highly depends on its potential to perform mammalian-like posttranslational modifications such as glycosylation. To gain more insight into the glycosylation potential of the baculovirus mediated insect cell expression system, we have studied the expression of glycosyltransferases involved in complex-type N-glycosylation. Lepidopteran insect cell lines derived from *Trichoplusia ni*, *Spodoptera frugiperda*, and *Mamestra brassicae* were found to express a β1 → 4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) that catalyzes the transfer of GalNAc from UDP-GalNAc to oligosaccharides and glycoproteins carrying a terminal β-linked GlcNAc residue. These results suggest that Lepidopteran insect cells are capable of synthesizing complex-type carbohydrate chains containing GalNAcβ1 → 4GlcNAc (LacdiNAc) units. Baculovirus infection of the cells, however, resulted in a decrease in the activity of β4-GalNAcT from 80 to <1 pmol·min^{-1} mg^{-1} protein within 48 h post infection. Furthermore, considerable β-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase activity was observed in insect cells, whether or not infected with baculovirus, as well as in the culture medium. These enzyme activities could be responsible for degradation of complex-type oligosaccharide chains containing LacdiNAc units. Our findings provide an enzymatic basis for the observation that most recombinant glycoproteins produced by baculovirus infected insect cells carry oligomannosidic-type N-linked glycans, in spite of the fact that uninfected insect cells have the potential for the synthesis of mammalian-like complex-type glycans.

Key words: acetylgalactosaminyltransferase/baculovirus/glycosyltransferase/hexosaminidase/Trichoplusia ni cells

Introduction

The carbohydrate moieties of glycoprotein therapeutics affect the biological activity, the stability, immunogenicity, and pharmacokinetics of these proteins (Cumming, 1991). The increasing importance of the production of such recombinant glycoproteins has raised wide interest in the glycosylation potential of heterologous expression systems.

Lepidopteran insect cells are popular as hosts for the expression of heterologous proteins by infection with recombinant baculoviruses. A large variety of mammalian proteins has been expressed in this system. The expression of foreign genes in baculovirus infected cells is mostly driven by the efficient viral polyhedrin promoter, which generally results in high yields of recombinant proteins compared to other eukaryotic expression systems (Luckow and Summers, 1988; Luckow, 1993). In addition, insect cells, like mammalian cells, are capable of both N- and O-glycosylation, and are therefore regarded as attractive hosts for the production of mammalian glycoproteins.

The biosynthesis of N-linked oligosaccharides in eukaryotes starts with the assembly of an oligomannosidic precursor oligosaccharide, which is transferred *en bloc* to a polypeptide. In vertebrates the oligosaccharide chain is then trimmed, and usually further processed to yield hybrid- or complex-type oligosaccharide chains (Schachter and Roseman, 1980). Little is known, however, about the potential for N-glycosylation in insect cells. Analysis of the carbohydrate chains of recombinant proteins produced in Lepidopteran insect cells often reveals the presence of simple mono- and disaccharide O-linked chains (Thomsen *et al.*, 1990; Chen *et al.*, 1991; Sugyiama *et al.*, 1993), and of oligomannosidic or truncated N-linked chains which sometimes are fucosylated (Jarvis and Summers, 1989; Kuroda *et al.*, 1990; Chen *et al.*, 1991; Voss *et al.*, 1993; Aeed and Elhammer, 1994; Kretzschmar *et al.*, 1994). These observations are consistent with results obtained in earlier studies on mosquito cells (Butters *et al.*, 1981; Hsieh and Robbins, 1984), in which evidence was obtained for the occurrence of the first processing steps leading to $Man_3GlcNAc_2$ glycans. Occasionally, however, the presence of complex-type oligosaccharide chains on recombinant proteins produced in Lepidopteran insect cells has been reported (Davidson *et al.*, 1990; Davidson and Castellino, 1991a,b). Information on glycosylation in other insect species is scarce, but complex-type N-glycosylation has been described to occur on phospholipase A_2 and hyaluronidase from honeybee venom (Kubelka *et al.*, 1993, 1995).

Several enzymes involved in the initiation of the synthesis of N-linked complex-type carbohydrate chains (the trimming enzymes α-mannosidase I and II, and the glycosyltransferases GlcNAc-transferase I and II) were shown to be active in Lepidopteran insect cells (Davidson *et al.*, 1991; Altmann *et al.*, 1993; Velardo *et al.*, 1993; Altmann and Marz, 1995; Ren *et al.*, 1995). In this context, it is not understood why foreign proteins expressed in these cells hardly ever carry complex-type glycans. One possible explanation for this observation is that enzymes responsible for the elongation reactions are normally not expressed in Lepidopteran cells. Alternatively, other processes may interfere with the elongation reactions.

More knowledge about the glycosylation potential in Lepidopteran insect cells and the conditions that allow the formation of mammalian-like complex-type carbohydrate chains on heterologous proteins would increase the value of the baculovirus/insect cell expression system for the production of mammalian glycoproteins. In this manuscript we report on the detection and enzymatic characterization of a UDP-GalNAc:GlcNAcβ-R β1 → 4-*N*-acetylgalactosaminyl transferase (β4-GalNAcT), in several Lepidopteran insect cell lines. Based on the acceptor specificity of the enzyme we conclude that the β4-GalNAcT is involved in the synthesis of Lac**di**NAc disaccharide units on complex-type N-glycans. However, baculovirus infection of the insect cells appears to interfere with this type of glycosylation.

Results

GalNAc-transferase activity in Trichoplusia ni cells

In a cell line derived from the Lepidopteran insect *T.ni* a GalNAc-transferase (GalNAcT) activity was detected, using assay conditions as described by Neeleman *et al.* (1994) with GlcNAc as the acceptor substrate. However, the product formed was rapidly broken down, presumably due to the action of β-*N*-acetylhexosaminidase(s) present in insect cells (Licari *et al.*, 1993; Bakker *et al.*, 1994). Therefore, optimal assay conditions were determined by varying the pH (pH 4–9) and the concentration of Triton X-100 and Mn^{2+}. Highest enzyme activity was measured in 0.1 M MOPS pH 8.0, containing 40 mM Mn^{2+} and 0.2% Triton X-100. Maximal β-*N*-acetylhexosaminidase activity was detected at pH 4.0. The β-*N*-acetylhexosaminidase activity in MOPS buffer at pH 8.0 was to 30–40% of the activity found in cacodylate buffer at pH 6.5, which was used before (Neeleman *et al.*, 1994). The addition of 100 mM GalNAc to the assay mixture further reduced breakdown of the product. In Figure 1 a comparison is shown of product formation and breakdown by the action of GalNAcT and β-*N*-acetylhexosaminidase(s), respectively, under different assay conditions. Clearly, the breakdown of the product as observed under suboptimal assay conditions (Figure 1A) does not allow an accurate determination of enzyme activity. Under these conditions most of the radioactivity migrates as free GalNAc (peak 2). In contrast, under optimal conditions the disaccharide product (peak 1) accumulates, and breakdown of this product is minimal (Figure 1B).

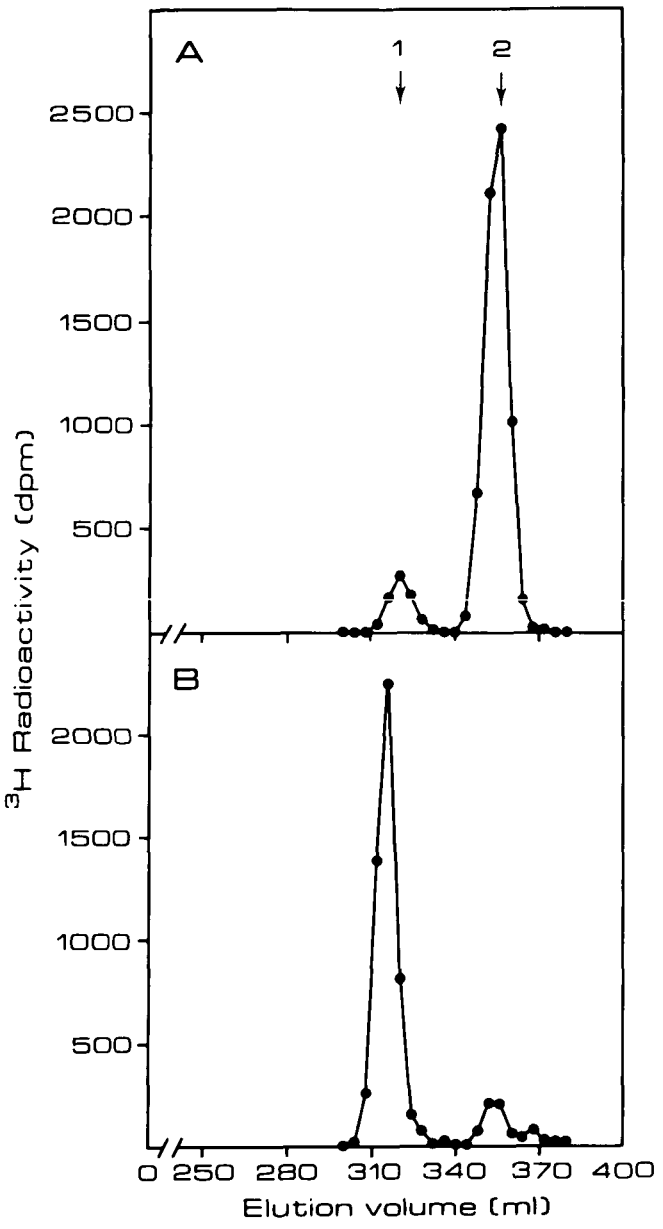

Fig. 1. Analysis by gel filtration of the products formed in an incubation mixture containing a cell extract of *T.ni* cells, UDP-[^{3}H]GalNAc as donor and GlcNAc as an acceptor. Incubation conditions and separation of the reaction products on a Bio-Gel P-4 column were as described in Materials and methods. Product peak 1 contained the disaccharide [^{3}H]GalNAc-GlcNAc, whereas peak 2 could be identified as [^{3}H]GalNAc. (**A**) Assay conditions (sodium cacodylate pH 6.5) as described by Neeleman *et al.* (Neeleman *et al.*, 1994). (**B**) Assay carried out in MOPS pH 8.0 containing 100 mM GalNAc.

GalNAcT and GalT activity in different Lepidopteran insect cell lines

GalNAcT and GalT activity was measured in cell lines derived from *Trichoplusia ni*, *Spodoptera frugiperda*, and *Mamestra brassicae* (Table I) using GlcNAc as an acceptor substrate. The results show that in all three cell lines both activities could be detected. The GalNAcT activity, however, was at least 10 times higher than the GalT activity. *T.ni* cells showed the highest GalNAcT activity but also the highest *N*-acetylhexosaminidase activity (results not

Table I. GalNAcT and GalT activity in different Lepidopteran insect cell lines; enzyme activities were assayed using GlcNAc (25 mM) as an acceptor substrate

Cell line	GalNAcT activity	GalT activity
	pmol·min^{-1}·mg^{-1} protein	
Trichoplusia ni (BTI-TN-5B1-4)	94	7
Spodoptera frugiperda (Sf9)	34	3
Mamestra brassica (IZD-Mb0503)	62	7

Table II. 400 MHz ^{1}H-NMR spectroscopic data of the product formed with GlcNAc as an acceptor by the action of GalNAc-transferase from *T.ni* cells

Reporter group	Residue	Anomer of compound	Chemical shift	Coupling constant
			ppm	*Hz*
H-1	GlcNAc	α	5.192 [5.191]	2.9 [2.9]
		β	4.701 [4.701]	7.5 [8.3]
	GalNAcβ4	α	4.530 [4.530]	8.5 [8.4]
		β	4.521 [4.521]	8.7 [8.5]
NAc	GlcNAc		2.037 [2.037]	
	GalNAcβ4		2.067 [2.066]	

The data are compared to known NMR spectroscopic values for GalNAcβ1→4GlcNAc (Neeleman *et al.*, 1994), that are indicated in brackets.

Table III. Acceptor specificity of β4-GalNAcT from *T.ni* cells. GalNAcT activity is expressed as a percentage of the activity obtained with 1 mM GlcNAc, which corresponded to 10 pmol·min^{-1}·mg^{-1} protein

Acceptor compound	Substrate concentration	Relative GalNAcT activity
	mM	%
1. GlcNAc	1	100
2. GlcNAc	25	600
3. GalNAc	1	<1
4. GalNAc	100	<1
5. GlcNAcα-O-pNP	1	<1
6. GlcNAcβ-O-pNP	1	330
7. GlcNAcβ-S-pNP	1	524
8. Galβ-O-pNP	1	5
9. Manα1→6 / Manα1→3 } Man	1	<1
10. GlcNAcβ1→6 / GlcNAcβ1→2 } Man	1	424
11. GlcNAcβ1→2 / GlcNAcβ1→4 } Man	1	307
12. GlcNAcβ1→2Manα1→6 / GlcNAcβ1→2Manα1→3 } Manβ1→4GlcNAc	1[a]	300
13. GlcNAcβ1→6 / Galβ1→3 } GalNAcα-O-pNP	1	370
14. GlcNAcβ1→3GalNAcα-O-pNP	1	313
15. asialo-agalacto-α_1-AGP	1[a]	76

[a] Substrate concentration expressed in terms of terminal GlcNAc residues.

shown). In Sf9 cells GalNAcT activity was lowest, and GalT activity was hardly detectable.

Product characterization

About 100 nmol of product, obtained by incubation of UDP-GalNAc with a *T.ni* cell extract as the enzyme source, and with GlcNAc as an acceptor, was analyzed by ^{1}H-NMR spectroscopy. The results in Table II show that the observed chemical shifts and coupling constants of the product are virtually identical to those obtained for the reference compound GalNAcβ1 → 4GlcNAc (Nemansky and Van den Eijnden, 1992; Neeleman *et al.*, 1994). Based on these data we conclude that the GalNAcT from *T.ni* cells is capable of catalyzing the transfer of a GalNAc residue from UDP-GalNAc in β1 → 4 linkage to terminal β-linked GlcNAc residues on acceptor molecules and thus can be defined as a UDP-GalNAc:GlcNAcβ-R β1 → 4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT).

Acceptor substrate specificity of T.ni β4-GalNAcT

GalNAcT assays were performed using a number of oligosaccharides and a glycoprotein as potential acceptor substrates (Table III). The results show that substrates carrying a terminal GlcNAc residue in β-configuration can serve as an acceptor for the GalNAcT. The oligosaccharide substrates **10**, **11**, and **12**, representing partial structures of N-linked glycans, as well as the substrates **13** and **14**, representing partial structures of O-linked glycans, appeared to be good acceptors. The product (mono-*N*-acetylgalactosaminylated) obtained with substrate **12** eluted in a HPAEC-PAD system with the same retention time as reference mono-*N*-acetylgalactosaminylated substrate **12**, obtained with the *Lymnaea stagnalis* β4-GalNAcT (Mulder *et al.*, 1995). Also the glycoprotein asialo/agalacto-α_1-AGP served as an acceptor, although it was less effective in comparison with the oligosaccharide substrates. Essentially no GalNAcT activity was observed using substrates with a terminal *N*-acetylgalactosamine, galactose or mannose residue, or a *N*-acetylglucosamine residue in α-anomeric configuration.

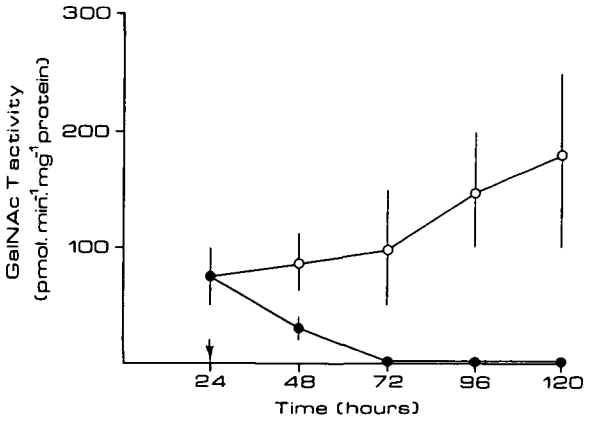

Fig. 2. GalNAcT activity in *T.ni* cells cultured up till 120 h. Culture medium was changed every 24 h. ○, Uninfected *T.ni* cells; ●, baculovirus infected cells. Baculovirus was added 24 h after seeding (↓). All values are expressed as the mean of two independent experiments, performed in duplicate.

The effect of culture conditions and baculovirus infection on the expression of β4-GalNAcT and N-acetylhexosaminidase(s)

To determine the optimal culture conditions for expression of β4-GalNAcT activity in *T.ni* cells, the cells were allowed to grow to different densities, with a change of medium every 24 h. Cells were collected at various times, and assayed for GalNAcT activity. Enzyme activity was maximal after culturing the cells for 90–120 h (Figure 2), when the cells had reached 100–150% confluency. The enzyme activity stayed at this level for a period of 48 h provided that the medium was changed daily. GalT activity appeared very low, varying from 2–7 pmol · min^{-1} · mg^{-1} protein (results not shown).

In addition, the influence of baculovirus infection on the β4-GalNAcT activity was studied. Cells were infected 24 h after seeding, and harvested at various times post infection. A considerable decrease in β4-GalNAcT activity was observed following infection (from 80 to <1 pmol · min^{-1} · mg^{-1} protein, Figure 2) and little or no β4-GalNAcT activity was detectable anymore 48 h post infection. By contrast, uninfected cells showed an increase in β4-GalNAcT activity. GalT activity was hardly detectable (<2 pmol · min^{-1} · mg^{-1} protein) at the time of infection as well as thereafter (results not shown).

In the same experiment, both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities were determined in the cell lysates and in the culture medium (Figure 3). Both enzyme activities were found to be similar. Cell associated β-*N*-acetylhexosaminidase activity showed little variation with the time both in uninfected and baculovirus infected cells (Figure 3A). During the course of the experiment, considerable β-*N*-acetylhexosaminidase activity continued to be released into the culture medium from uninfected as well as from baculovirus infected cells (Figure 3B).

Discussion

Lepidopteran insect cells are widely used as host cells for the production of foreign proteins, encoded by recombinant baculoviruses. Up till now surprisingly little is known about the glycosylation potential of insect cells, which limits certain applications of this system. Lepidopteran insect cells contain enzymes involved in the first steps of complex-type N-glycosylation (Davidson *et al.*, 1991; Altmann *et al.*, 1993; Velardo *et al.*, 1993; Altmann and Marz, 1995; Ren *et al.*, 1995). However, no glycosyltransferases involved in the extension or the termination of complex-type carbohydrate chains have been detected so far. We therefore decided to examine the presence and the expression characteristics of these glycosyltransferases.

Three alternative pathways have been described for the synthesis of complex-type glycans. In the most common pathway Galβ1 → 4GlcNAc (LacNAc) structural units are formed by the action of β4-galactosyltransferase (β4-GalT). This route is frequently found in mammalian species. In addition, mammalian glycoprotein glycans have been identified that contain GalNAcβ1 → 4GlcNAc (Lac**di**NAc) disaccharide units. These structures are also found in several non-mammalian species as schistosomes, nematodes, molluscs, insects, snakes, or amphibians (reviewed in Van den Eijnden *et al.*, 1995b). They are formed by the action of a β1 → 4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) in a second pathway of complex-type glycan synthesis (Smith and Baenziger, 1988; Neeleman *et al.*, 1994; Srivatsan *et al.*, 1994; Mulder *et al.*, 1995). A third pathway, leading to the formation of non-core GlcNAcβ1 → 4GlcNAc structural units in complex-type glycans, has recently been proposed (Bakker *et al.*, 1994; Van den Eijnden *et al.*, 1995a). Up till now, the latter pathway only has been suggested to occur in the snail *Lymnaea stagnalis* and the snake *Bothrops moojeni* (Bakker *et al.*, 1994; Lochnit and Geyer, 1995).

Here we report on the detection of a GalNAc-transferase in three Lepidopteran insect cell lines derived from *Trichoplusia ni*, *Spodoptera frugiperda*, and *Mamestra brassicae* respectively. Characterization of the enzyme product revealed that the enzyme can be defined as a UDP-GalNAc:GlcNAcβ-R β1 → 4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT). A similar β4-GalNAcT activity has been found in schistosomes (Neeleman *et al.*, 1994; Srivatsan *et al.*, 1994), and in the albumen gland of the pond snail *Lymnaea stagnalis* (Mulder *et al.*, 1995). Like these enzymes, the insect β4-GalNAcT transfers a GalNAc residue to acceptors containing a terminal β-linked *N*-acetylglucosamine residue. Our results indicate that the insect cell enzyme may be involved in the biosynthesis of Lac**di**NAc disaccharide units in both N- and O-linked complex-type glycans. Since, however, no O-linked structures containing a β-GlcNAc have been described on insect glycoproteins so far, the action of the β4-GalNAcT may be confined to N-linked glycans in these cells.

In addition, we detected a minor β4-GalT activity. As this enzyme activity was quite low, the relevance of this observation is not yet clear. The activity might be due to a flexibility in the donor substrate requirement of the β4-GalNAcT, as has been documented to occur for β4-GalT with UDP-GalNAc (Palcic and Hindsgaul, 1991). Alternatively, it might be possible that insect cells are capable of synthesizing LacNAc units in complex-type glycans. In that case, however, the β4-GalT activity is expected to compete with the β4-GalNAcT activity for the same glycoconjugate substrates, as both enzymes show a similar acceptor specificity (Blanken *et al.*, 1984; Schachter and Roseman, 1980).

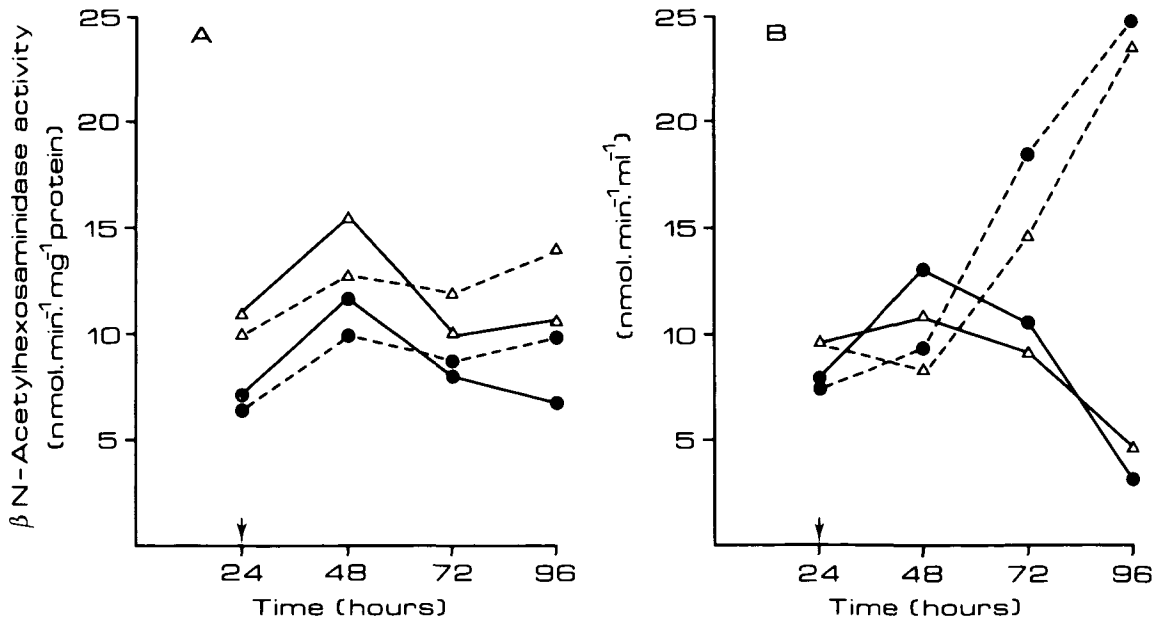

Fig. 3. β-*N*-Acetylhexosaminidase activity in *T. ni* cells (**A**) and culture medium (**B**). The cells were grown for 120 h during which period the culture medium was changed every 24 h. β-*N*-Acetylhexosaminidase activity was assayed using GalNAcβ-O-pNP (●) or GlcNAcβ-O-pNP (△) as the substrate. The dotted lines represent uninfected cells, the solid lines baculovirus infected cells. Baculovirus was added 24 h after seeding (↓). All values are expressed as the mean of two independent experiments, showing a deviation of the mean of <10%.

As the β4-GalNAcT levels as present in Lepidopteran insect cells have been shown to be 10 times higher than the β4-GalT levels, it is expected that Lac**di**NAc rather than LacNAc containing oligosaccharide chains will be formed in these cells. Up till now, structural data of N- and O-linked glycans of endogenous Lepidopteran glycoproteins are lacking. In honeybee venom, however, Lac**di**NAc-containing N-linked glycans indeed have been described, indicating that β4-GalNAcT is functional in insects (Kubelka *et al.*, 1993, 1995). It thus appears from structural and enzymatic data that insect cells have the potential of assembling complex-type, Lac**di**NAc-type oligosaccharides on glycoproteins.

Baculovirus infection interferes with complex-type glycosylation

While no structural data are available of endogenous glycans derived from insect cells in culture, the glycans of heterologous recombinant proteins produced in these cells have been studied by several groups. Interestingly, complex-type glycans have so far been reported to occur only on recombinant human plasminogen produced in Lepidopteran insect cell lines (Davidson *et al.*, 1990; Davidson and Castellino, 1991a,b). These glycans contained LacNAc, rather than Lac**di**NAc disaccharide units, and it was suggested that such complex-type glycans were formed by an induction or activation of the relevant glycosyltransferases as a consequence of baculovirus infection (Davidson and Castellino, 1991a). We therefore assayed the activities of both GalNAcT and GalT at different periods of time after baculovirus infection. It was found that the β4-GalNAcT activity rapidly decreased as a consequence of baculovirus infection, and became non-detectable within 48 h after infection. The β4-GalT activity was much lower at the time of infection and similarly became nondetectable. These observations do not support the suggestion of Davidson and Castellino (1991a), that glycosyltransferases are induced upon infection. They rather are in line with studies of Altmann *et al.* (1993), who could not detect an increase in the levels of GlcNAc-transferase I or II after baculovirus infection.

Expression of foreign proteins in insect cells generally occurs under transcriptional control of the strong polyhedrin promoter, that acts very late in the infection process. The decrease of glycosyltransferase activity we observed provides an explanation for the fact that recombinant proteins produced in baculovirus infected insect cells usually do not carry complex-type oligosaccharide chains. Our results would suggest that expression of a recombinant protein under transcriptional control of an early baculovirus promoter might increase the chance of obtaining recombinant proteins carrying complex-type, lac**di**NAc containing, carbohydrate chains.

β-N-Acetylhexosaminidase activity in Lepidopteran insect cell lines

Considerable β-*N*-acetylglucosaminidase activity was observed by us and others (Licari *et al.*, 1993; Altmann *et al.*, 1995) both within the cells and in the culture medium. In addition, we detected similar levels of a β-*N*-acetylgalactosaminidase activity. In contrast to what was found for the β4-GalNAcT activity in the cells, baculovirus infection caused only a minor decrease of β-*N*-acetylhexosaminidase activity in the cells, and the activity continued to accumulate in the culture medium for at least 90 h post infection. Recently, Altmann *et al.* (1995) detected a membrane-bound β-*N*-acetylhexosaminidase in several insect cell lines. Their results suggest that this enzyme probably is

involved in the processing of protein N-linked glycans. The β-*N*-acetylhexosaminidases secreted in the culture medium might cause degradation of the oligosaccharide chains of glycoproteins. Recombinant proteins that are secreted into the culture medium will be susceptible to the action of the β-*N*-acetylhexosaminidases, but also proteins that are expressed intracellularly will be affected, as the baculovirus infection is lytic. The β-*N*-acetylhexosaminidase action is expected to be very unfavourable for the formation of Lac**di**NAc containing oligosaccharide chains; both GlcNAcβ → R, the acceptor for β4-GalNAcT, and the *N*-acetylgalactosaminylated product formed by the action of this enzyme might be susceptible to degradation. Therefore, the observed secretion of β-*N*-acetylhexosaminidases seems a drawback of using these cells in culture for the production of recombinant glycoproteins.

Complex-type glycosylation of Lepidopteran insect cell glycoproteins

In summary, the present work shows that Lepidopteran insect cells in tissue culture posses the enzymatic capacity for the synthesis of mammalian-like complex-, Lac**di**NAc-type carbohydrate chains. It is unknown, due to the lack of knowledge regarding the carbohydrate structures on endogenous insect cell glycoproteins, whether the enzyme indeed plays a role in protein glycosylation in cell culture. However, it is likely that this is the case, based on structural data available for glycoproteins found in whole insects (Kubelka *et al.*, 1993, 1995). On the other hand, the β-*N*-acetylhexosaminidases secreted by insect cells in tissue culture may remove terminal β-GlcNAc and β-GalNAc residues on the cell surface.

We would anticipate that β4-GalNAcT acts also on recombinant glycoproteins produced in insect cell cultures. However, the observed decrease in β4-GalNAcT activity upon baculovirus infection is expected to result in a strongly decreased biosynthesis of Lac**di**NAc containing glycans. The low β4-GalNAcT activity combined with an accumulation of β-*N*-acetylhexosaminidases in the medium makes it very unlikely that in baculovirus-infected insect cells in culture, glycoproteins will be formed with Lac**di**NAc complex-type glycans.

Materials and methods

Materials

GlcNAc, GalNAc, compounds **5–8** (see Table III) and p-nitrophenyl-*N*-acetyl-β-D-galactosaminide (GalNAcβ-O-pNP), were obtained from Sigma. Compounds **13** and **14** were obtained from Toronto Research Chemicals (Toronto, Ontario). Compounds **9**, **10**, and **11** were kindly supplied by Dr. J. Lönngren (University of Stockholm, Stockholm, Sweden). The β4-galactosylated form of compound **12** was a gift of G. Strecker (Université des Sciences et Techniques de Lille Flandres-Artois, Villeneuve d'Ascq, France). UDP-[^{3}H]GalNAc (New England Nuclear) was diluted with unlabelled UDP-GalNAc (Sigma) to give the desired specific radioactivity. Morpholinopropane sulfonic acid (MOPS) was obtained from Serva. α_1-Acid glycoprotein (α_1-AGP) was isolated from human plasma Cohn fraction V supernatant according to Hao and Wickerhauser (Hao and Wickerhauser, 1973). Asialo-α_1-AGP was desialylated by treatment with 0.1 M trifluoroacetic acid for 1 h at 80°C. Degalactosylation was performed by incubation with jack bean β-galactosidase (0.2 U/mmol terminal Gal) in 50 mM sodium acetate pH 4.0 to obtain the asialo-agalacto forms.

Maintenance and infection of insect cells

Trichoplusia ni (*T. ni* 'BTI-TN-5B1-4') and *Spodoptera frugiperda* pupal ovary tissue (Sf9) insect cells were obtained from Invitrogen. The *Mamestra brassica* cell line IZD-Mb0503 was established at the Technical University of Darmstadt (Darmstadt, Germany) and was kindly provided by Dr. H.G.Miltenburger. All cells were grown as monolayers at 27°C in Hink's TNM-FH medium (JRH Biosciences) supplemented with 10% fetal calf serum (Gibco-BRL) and 50 μg/ml gentamycine. For the time course experiments, *T.ni* insect cells were seeded in 6-well plates (Greiner) at $3 \cdot 10^5$ cells per 9.6 cm^2-well. Twenty-four hours after seeding, when the cells showed 50–60% confluency, the medium was replaced by 2 ml of fresh, serum-free Excell-401 medium (JRH Biosciences) containing 50 μg/ml gentamycine. To half of the wells, baculovirus was added (multiplicity of infection of 5); the others served as controls. Every 24 h the cells and medium of one well were collected for enzyme assays. The medium was centrifuged for 5 min at 1500 r.p.m. in an Eppendorf centrifuge to remove detached cells (which were added to the cell suspension). The cells of one well were suspended in 1 ml of PBS and collected by centrifugation. The cell pellet was resuspended in 150 ml of 50 mM MOPS pH 8.0/0.5% Triton X-100, and the cell lysate was used immediately in the enzyme assays. The medium of the remaining cultures was changed daily.

Preparation of the enzyme and glycosyltransferase assays

After insect cells had been grown in Hink's medium for 48 h (80–90% confluency), the medium was replaced by serum free Excell medium and the cell culture was continued for an additional 24 h to >100% confluency. Subsequently, the cells were washed with PBS, incubated for 20 min in 50 mM MOPS pH 8.0/0.5% Triton X-100 at 4°C, scraped off the flask, and collected in an Eppendorf vial. The mixture was vortexed for 10 s, and centrifuged to remove cell debris. The supernatant (cell extract) was used as the enzyme source in glycosyltransferase assays.

Standard GalNAc-transferase assays were performed in a 50 μl reaction mixture containing 5 μmol of MOPS pH 8.0, 25 nmol of UDP-[^{3}H]GalNAc (approximately 1 Ci/mol), 2 μmol of $MnCl_2$, 0.2 μmol of ATP, 0.1 μl of Triton X-100, 15 μl of cell extract, and 1.25 μmol of GlcNAc. For acceptor specificity studies, acceptor substrate concentrations were as indicated in Table III. Unless indicated otherwise, 5 μmol of GalNAc was included in the reaction mixture to inhibit the action of β-*N*-acetylhexosaminidase(s). Control assays lacking the acceptor substrate were carried out to correct for incorporation into endogenous acceptors. After incubation at 37°C for 60–120 min the reaction was stopped. When GlcNAc was the acceptor, the labeled product was separated from unincorporated label by chromatography on a 1 ml column of Dowex 1-X8 (Cl^- form) according to Easton *et al.* (1992). Where indicated, the flowthrough was subsequently fractionated (separation of GalNAc from GalNAc-GlcNAc) on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh, Bio-Rad) run in 50 mM ammonium acetate pH 5.2 to estimate breakdown of product during the incubation. When pNP substrates were used as the acceptor, the product was isolated using Sep-pak C-18 cartridges (Waters) as described by Palcic *et al.* (1988).

Galactosyltransferase assays were carried out in a 50 μl reaction mixture containing 5 μmol of MOPS pH 8.0, 25 nmol of UDP-[^{3}H]Gal (1 Ci/mol), 1 μmol of $MnCl_2$, 0.2 μmol of ATP, 0.25 μl of Triton X-100, 1.25 μmol of GlcNAc, and 15 μl of cell extract. In this buffer, the β4-GalT activity was similar to that measured under standard β4-GalT assay conditions (Blanken *et al.*, 1982).

β-N-Acetylhexosaminidase assays

β-*N*-Acetylgalactosaminidase and β-*N*-acetylglucosaminidase assays were carried out in a 200 μl reaction mixture containing 20 μmol of citric acid/40 μmol of disodium hydrogen phosphate pH 4.5, 1 μl of cell extract or 5 μl of medium, and 200 nmol of GalNAcβ-O-pNP or GlcNAcβ-O-pNP respectively. The mixtures were incubated for 60–90 min at 37°C. The reaction was terminated by adding 1 ml of a solution containing 1 mmol of sodium carbonate. Liberated pNP was determined by measuring the absorbance at 420 nm using a Shimadzu spectrofotometer.

Product identification

The standard incubation mixture was scaled up 50-fold and incubated for 2.5 h at 37°C. The product was separated from UDP-[^{3}H]GalNAc by ion exchange on a column of Dowex 1-X8 (Cl^-), isolated on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) run in 50 mM ammonium

acetate pH 5.2, and desalted on a column (0.7 × 45 cm) of Bio-Gel P-2 (200–400 mesh) run in water. The sample was treated with D_2O (99.75 atom% (Merck)) three times with intermediate lyophilization. Finally, the sample was redissolved in 400 μl of D_2O (99.95 atom% (Aldrich)). ^{1}H-NMR spectroscopy was performed at 400 MHz at a probe temperature of 300°K on a Bruker MSL-400 spectrometer (Department of Physics, Vrije Universiteit, Amsterdam) operating in the Fourier-transform mode. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation. Chemical shifts are expressed downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm).

HPLC analysis of N-acetylgalactosaminylated product

N-Acetylgalactosaminylated compound **12** was analyzed by HPLC. The HPAEC-PAD system used consisted of a Dionex Bio-LC gradient pump, a CarboPac PA-100 column (4 × 250 mm), and a Model PAD 2 detector. The following pulse potentials and durations were used for detection: E_1 = 0.05 V (t_1 = 480 ms), E_2 = 0.60 V (t_2 = 120 ms), E_3 = −0.60 V (t_3 = 60 ms). The response time of the detector was set to 1 s. For the separation of the products, isocratic elution was conducted with 0.1 mol/litre NaOH for 10 min, whereafter a gradient of sodium acetate (0–0.1 mol/liter in 40 min) in 0.1 mol/litre NaOH was applied. The flow rate was 1 ml/min, and fractions of 0.5 ml were collected and counted for radioactivity.

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Abbreviations

β4-GalNAcT, UDP-GalNAc:GlcNAcβ-R β1 → 4-*N*-acetylgalactosaminyltransferase; LacNAc, N-acetyllactosamine (Galβ1 → 4GlcNAc); Lac**di**NAc, *N,N'*-diacetyllactosediamine (GalNAcβ1 → 4GlcNAc); α_1-AGP, α_1-acid glycoprotein; pNP, para-nitrophenol; GlcNAc-transferase, *N*-acetylglucosaminyltransferase.

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