Sialylation of N-Glycans on the Recombinant Proteins Expressed by a Baculovirus-Insect Cell System under β-N-Acetylgalcosaminidase Inhibition*

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We investigated the ability of a baculovirus-insect cell system to produce sialylated glycoproteins. Despite the presence of enzymes for synthesizing complex-type N-glycans, the most frequent structure of insect N-glycan is the paucimannosidic type, Manα1,2GlcNAcβ1,3(Fuc). The reason for the overwhelming assembly of paucimannosidic N-glycans is not yet well understood. We hypothesized that this predominance might be due to insect-specific, Golgi-associated β-N-acetylgalcosaminidase (GlcNAcase)-mediated removal of N-acetylgalcosamine residues from the precursor N-glycan, thereby preventing its galactosylation and terminal sialylation. As we expected, the suppression of intrinsic GlcNAcase activity with a specific inhibitor, 2-acetamido-1,2-dideoxyxyojirimycin, allowed the accumulation of sialylated glycoproteins in the supernatants of insect cell cultures after baculoviral infection. Our observation indicates that GlcNAcase-dependent depletion of N-acetylgalcosamine residues from intermediate N-glycans is critical for the assembly of paucimannosidic N-glycans in insect cells and, more importantly, that insect cells (under specific conditions) retain the ability to construct sialylated N-glycans like those in mammalian cells.

Insect cells have similarities to mammalian cells with regard to posttranslational events such as proteolytic processing (1), phosphorylation of serine residues (2, 3), and C-terminal amidation (4). These properties of insect cells are thought to be useful for the production of recombinant mammalian proteins by baculovirus-insect cell systems because the produced proteins are expected to be structurally and biologically equivalent to native ones. In addition to these posttranslational events, glycosylation (particularly N-glycosylation and terminal sialic acid modification) is important for the biological activity and stability of synthesized proteins (5–8). In mammalian cells, the N-glycosylation pathway starts by the transfer of a dolichol-linked precursor oligosaccharide (Glc3Man9GlcNAc5) to a nascent polypeptide. Then, this precursor oligosaccharide is sequentially processed by glycosidases and glycosyltransferases, such as α-mannosidase I, α-mannosidase II, acetylgalcosaminyltransferase I, acetylgalcosaminyltransferase II, fucosyltransferase, and galactosyltransferase. The resulting N-glycan is finally sialylated by sialyltransferase at the trans-Golgi (9). The precursor oligosaccharide and the requisite enzymes (except sialyltransferase) have been identified previously in insect cells (10–18). Although these findings suggest the presence of an N-glycosylation pathway in insect cells similar to that seen in mammalian cells, the most frequent structure of insect N-glycans is the paucimannosidic type, Manα1,2GlcNAcβ1,3(Fuc), which is not found in mammalian cells (19–21). One striking difference between insect and mammalian N-glycosylation pathways is the biological property of β-N-acetylgalcosaminidase (GlcNAcase) activity. Mammalian GlcNAcase is localized in a soluble fraction of lysosomes and hydrolyzes β-linked terminal GlcNAc residues on degrading glycoconjugates (22). In contrast, insect GlcNAcase is detected in both soluble and membrane-associated fractions and hydrolyzes β-1,2-linked terminal GlcNAc residues only from α-1,3-bound mannose cores (23). This membrane-associated GlcNAcase has been reported to be located at the Golgi (24). We hypothesized that the insect-specific, membrane-associated GlcNAcase may play a key role in the synthesis of paucimannosidic N-glycans by preventing the galactosylation and subsequent sialylation of expressed glycoproteins.

To prove our hypothesis, we produced recombinant glycoproteins by using a baculovirus-insect cell system in the presence of a GlcNAcase inhibitor and determined the terminal carbohydrate structure of these glycoproteins.

EXPERIMENTAL PROCEDURES

Reagents—A mouse monoclonal antibody (mAb) against bovine interferon τ (boIFN-τ) was kindly provided by Katakura Industries (Saitama, Japan). The GlcNAcase inhibitor, 2-acetamido-1,2-dideoxyxyojirimycin (2-ADN), was purchased from Tront Research Chemicals (North York, Ontario, Canada). p-Nitrophenyl-β-D-N-acetylgalcosaminide was purchased from Seikagaku Kogyo (Tokyo, Japan). Horseradish peroxidase-labeled Sambucus nigra agglutinin (SNA) was obtained from EY Laboratory (San Mateo, CA). Arthrobacter ureafaciens sialidase was purchased from Roche Molecular Biochemicals. 3′-Sialyllactose and 6′-sialyllactose were purchased from Sigma.

Recombinant Baculoviruses and Expression of Recombinant Proteins—To obtain boIFN-τ cDNA, template mRNA was prepared from primary bovine trophoblast cells as described previously (25), and

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1 The abbreviations used are: GlcNAcase, β-N-acetylgalcosaminidase; GlcNAc, N-acetylgalcosamine; boIFN-τ, bovine interferon τ; rboIFN-τ, recombinant bovine interferon τ; 2-ADN, 2-acetamido-1,2-dideoxyxyojirimycin; mAb, monoclonal antibody; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; SNA, Sambucus nigra agglutinin.
cDNA encoding the open reading frame of boIFN-τ was amplified by reverse transcription-PCR and then cloned into plasmid vector pCRII (Invitrogen). The cDNA was excised and inserted into the baculovirus transfer vector (pAcYM1) to obtain the recombinant transfer vector, pAcBIFNτ. The recombinant baculovirus, AcBIFNτ, was then established by using pAcBIFNτ according to a method described previously (26). The other recombinant baculoviruses, AcBGM (27) and AcPIL2 (28), which encode bovine GM-CSF and porcine interleukin (IL)-2, respectively, were obtained similarly. To produce the recombinant proteins, the Trichoplusia ni-derived cell line BTI TN 5B1-4 (TN5) was infected with recombinant baculovirus at a multiplicity of infection of 1.0 and then cultured in serum-free EX-CHEL401 medium (JRH Biosciences, Lenexa, KS) at 28 °C for 3 days. The culture supernatants were harvested, clarified by centrifugation, and stored at −20 °C until use.

**Inhibition of N-Glycosylation and GlcNAcase Activity**—To inhibit N-glycosylation, recombinant baculovirus-infected TN5 cells were cultured in medium containing 1 μg/ml tunicamycin (Sigma) for 3 days. To suppress GlcNAcase activity, TN5 cells were preincubated in medium containing 0–5 mM 2-ADN for 3 days, inoculated with the recombinant baculovirus, and then cultured for another 3 days in the presence of 2-ADN at the same concentration used in the preinculture.

**Electrophoresis and Western Blot Analysis**—Aliquots (20 μl each) of the supernatants were subjected to tricine-SDS-PAGE under reducing conditions (29). After electrophoresis, separated proteins were stained with Coomassie Brilliant Blue G-250 or transferred to polyvinylidene difluoride membrane (Millipore). The membrane was then blocked with 5% skim milk in phosphate-buffered saline overnight at 4 °C. The treated membrane was sequentially probed with 0.2 μg/ml anti-boIFN-τ mAb and horseradish peroxidase-conjugated rabbit anti-mouse IgG (1: 5000) at room temperature for 1 h each. Reacted proteins were made visible by 3,3’-diaminobenzidine staining.

**Lectin Blot Analysis**—The supernatants (600 ng recombinant protein/lane) were subjected to tricine-SDS-PAGE under reducing conditions, and the separated proteins were transferred to polyvinylidene difluoride membrane. The membrane was blocked with 1% bovine serum albumin in phosphate-buffered saline overnight at 4 °C. The treated membrane was washed with 0.05% (v/v) Tween 20 in 200 mM Tris-buffered saline. The membrane was probed with 5 μg/ml horseradish peroxidase-labeled SNA for 1 h at room temperature. Reacted carbohydrates were made visible on x-ray film by using the Renaissance Western blot Chemiluminescence Kit (PerkinElmer Life Sciences).

To confirm the specificity of SNA, we incubated the membrane with the transferred proteins in 0.02 M citrate buffer (pH 5.0) alone or with sialidase (0.5 unit/200 μl) for 20 h at 37 °C. In addition, we preincubated the horseradish peroxidase-labeled SNA with 10 mM 2-methyl-1-L-ascorbic acid and the absorbance at 420 nm (indicative of the transferred proteins in 0.02 M citrate buffer (pH 5.0)) was measured. The membrane was then blocked with 5% skim milk in phosphate-buffered saline overnight at 4 °C. The treated membrane was washed with 0.05% (v/v) Tween 20 in 200 mM Tris-buffered saline. The membrane was probed with 5 μg/ml horseradish peroxidase-labeled SNA for 1 h at room temperature. Reacted carbohydrates were made visible on x-ray film by using the Renaissance Western blot Chemiluminescence Kit (PerkinElmer Life Sciences).

**RESULTS**

**Expression of rboIFN-τ—boIFN-τ** is a glycoprotein containing 196 amino acids with a potential N-glycosylation site at Asn-78. Thus, boIFN-τ is an ideal molecule for analysis of the N-glycosylation process in insect cells. rboIFN-τ was expressed by TN5 cells infected with AcBIFNτ. Proteins of ~19.5 and 22 kDa were secreted and accumulated in the culture supernatant (Fig. 1A). Western blot analysis using anti-boIFN-τ mAb showed that these two molecules were structurally related (Fig. 1B). At ~19.5 kDa, the glycoprotein was detected by Coomassie Brilliant Blue staining and Western blot analysis (Fig. 1, A and B, lane 3). These results indicate that the 22-kDa molecule was N-glycosylated, whereas the 19.5-kDa molecule was not.

**Effects of a GlcNAcase Inhibitor on the Glycosylation of rboIFN-τ**—Because Woynarowska et al. (30) reported that maximum inhibition (~80%) of mammalian GlcNAcase was achieved by using 5 mM 2-ADN, we initially used this concentration. To examine the effect of GlcNAcase inhibition on expression of rboIFN-τ, TN5 cells were treated with 2-ADN as described under “Experimental Procedures.” We found that 5 mM 2-ADN did not affect cell growth (data not shown) or the quantity of expressed rboIFN-τ (Fig. 2A). The molecular mass of N-glycosylated rboIFN-τ increased from 22 to 23.3 kDa, but that of non-N-glycosylated rboIFN-τ (19.5 kDa) did not (Fig. 2). This result indicates that the shift in the molecular mass was due to an increase of oligosaccharide moiety.

To investigate the difference between the oligosaccharide structures of the 22- and 23.3-kDa molecules, we produced rboIFN-τ in the presence of various concentrations of 2-ADN and examined the terminal structures of the rboIFN-τ N-glycans by lectin blot analysis. The GlcNAcase activity in these cells decreased proportionally to the dose of 2-ADN, and 79.1% ± 1.2% (mean ± S.D.) of the activity in TN5 cells was inhibited at 5 mM 2-ADN, as was reported to occur in mammalian cells (Fig. 3A) (30). SNA, which recognizes α-2,6-linked sialic acids, reacted with the 23.3-kDa glycoprotein expressed in the presence of more than 0.5 mM 2-ADN, and the intensity of the SNA-reactive bands increased proportionally to the dose of 2-ADN without affecting total amounts of expressed rboIFN-τ (Fig. 3, B and C). The reaction of SNA with rboIFN-τ was diminished by treatment with sialidase (Fig. 4, A and B) or by preabsorption of SNA with 6′-sialyllactose but not with 3′-sialyllactose (Fig. 4, C and D). In addition, *Maackia amurensis* agglutinin lectin, which recognizes α-2,3-linked sialic acid, did not react with rboIFN-τ (data not shown). These findings indicate that α-2,6-linked sialic acid but not α-2,3-linked sialic acid is present at the terminus of the N-glycan on rboIFN-τ expressed under GlcNAcase-inhibited conditions.

**Assembly of Sialylated N-Glycans by Insect Cells**

**Sialylation on Other Recombinant Proteins**—To examine the effect of GlcNAcase inhibition on the assembly of N-glycans on other recombinant glycoproteins, we produced recombinant bovine GM-CSF and recombinant porcine IL-2 in the presence of 5 mM 2-ADN and performed lectin blot analysis with SNA. The molecular masses of glycosylated recombinant bovine GM-CSF and recombinant porcine IL-2 expressed by TN5 cells markedly increased in the presence of 5 mM 2-ADN (Fig. 5A), and SNA reacted with the molecules expressed under GlcNAcase-inhibited conditions (Fig. 5B, lanes 1 and 3). These results demonstrate that the α-2,6-linked terminal sialylation occurred not only on rboIFN-τ but also on other recombinant proteins expressed under the same conditions.

**DISCUSSION**

We produced bovine interferon τ, bovine GM-CSF, and porcine IL-2 in the presence of 2-ADN by using a baculovirus-
assembly of sialylated n-glycans by insect cells

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Fig. 2. Expression of rboIFN-τ under GlcNAcase-inhibited conditions. The supernatants of baculovirus-infected TN5 cells in the presence (+) or absence (−) of 5 mM 2-ADN were separated by tricine-SDS-PAGE and stained with Coomassie Brilliant Blue (A) or anti-boIFN-τ mAb (B). Wild-type virus (lane 1); AcBIFN-τ (lanes 2 and 3). Molecular mass markers are indicated on the left.

Fig. 3. Effects of 2-acetamido-1,2-dideoxyojirimycin on the expression of sialylated rboIFN-τ. A, inhibition of p-nitrophenol formation of TN5 cell GlcNAcase by 2-ADN. TN5 cell lysates were mixed with 0, 0.005, 0.05, 0.5, 1, 2, or 5 mM 2-ADN (lanes 1–7, respectively). Each point represents the mean ± S.D. from two independent experiments performed in triplicate. B, detection of sialic acids on rboIFN-τ by lectin blot analysis with SNA. rboIFN-τ was expressed by TN5 cells in the presence of 2-ADN at the same concentrations as described for A. C, Coomassie Brilliant Blue staining.

Fig. 4. Confirmation of the α-2,6-linked sialylation of rboIFN-τ expressed by TN5 cells. The rboIFN-τ expressed in the presence (+) or absence (−) of 5 mM 2-ADN was analyzed by lectin blotting with SNA. The membrane was treated with buffer alone (A) or with sialidase (B) before the SNA staining. Untreated membrane was probed with SNA preincubated with 0.1 mM 6'-sialyllactose (C) or 10 mM 3'-sialyllactose (D). Wild-type virus (lane 1); AcBIFN-τ (lanes 2 and 3). insect cell system, and we investigated the N-glycan structure of these recombinant molecules by using lectin blot analysis. It is widely known that the major structure of the N-glycans on insect glycoproteins is Man₃GlcNAc₂(±Fuc), which may be derived from GlcNAcMan₃GlcNAc₂ through mannosidase-mediated excision of α-linked mannosyl residues, followed by GlcNAcase-mediated depletion of a β-1,2-linked terminal GlcNAc residue (Fig. 6).

The distribution of GlcNAcase activity in insect cells has been reported to be unique. Whereas mammalian GlcNAcase activity is detectable only in a lysosomal compartment, the activity of insect cells is detected in a membrane-associated fraction as well as in the cytosol (23). Marchal et al. (24) reported that GlcNAcase activity in insect cells was located at the Golgi in the form of a membrane-bound molecule. This insect-specific GlcNAcase hydrolyzes β-1,2-linked GlcNAc residues on α-1,3-bound mannosyl cores in a strict manner, and the temporal formation of such GlcNAcase-susceptible structures in the N-glycosylation pathway has been reported (24). Therefore, we hypothesize that the activity might be involved in the removal of β-1,2-linked GlcNAc residues from intermediate N-glycans on their way to the formation of complex type N-glycans. As we expected, suppression of the intrinsic GlcNAcase activity with 2-ADN induced the extension and terminal sialylation of N-glycans on recombinant proteins expressed by baculovirus-infected insect cells (Figs. 4 and 5). The observation that the accumulation of sialylated proteins was inversely correlated with GlcNAcase activity in TN5 cells confirmed our hypothesis (Fig. 3). Wagner et al. (31) reported the increase of β-1,2-linked terminal GlcNAc residues on influenza hemagglutinin expressed by baculovirus-infected SF9 cells under GlcNAc-inhibited conditions. Although these investigators did not examine the intrinsic galactosylation and sialylation activities of those cells, their observation suggested that the inhibition of GlcNAcase prevented the processing of paucimannosidic structures and allowed the accumulation of substrates for the synthesis of complex-type glycans.

Despite many investigators’ attempts to identify sialyltransferase activity in insect cells, this issue had remained unclear. In the present study, we demonstrated the presence of α-2,6-linked sialic acid but not α-2,3-linked sialic acid at the terminus of the rboIFN-τ N-glycan synthesized by baculovirus-infected cells under GlcNAcase-inhibited conditions. We obtained a similar result for the N-glycans of recombinant bovine GMI-CSF and recombinant porcine IL-2 synthesized under the same conditions. In an experiment using the Spodoptera frugiperda-derived SF21AE and Bombyx mori-derived Bm-N insect cell lines, we found sialylation of N-glycans on the recombinant proteins expressed under the same conditions (data not shown). These findings indicate that those insect cell lines contain a significant α-2,6-sialyltransferase activity even at low levels. Thus, we considered that an insect cell line of lepidopteran origin would be able to synthesize sialylated N-glycans on any glycoprotein when GlcNAcase is inhibited. As we discussed previously, the treatment of insect cells with 2-ADN would enhance the accumulation of substrates possess-
Researchers have attempted to modify baculovirus-insect cell coproteins when they are used in vivo following a β,1-2-linked GlcNAc residue, thereby leading to further processing by galactosyltransferase and sialyltransferase to form sialylated N-glycans. In contrast, we did not observe terminal sialylation under conventional (non-GlcNAcase-inhibited) culture conditions. Previous studies on recombinant human plasminogen demonstrated the presence of α-2,6-linked sialic acids on recombinant human plasminogen expressed by a similar system under conventional culture conditions (32). Although the reason for the apparently discrepant results between recombinant human plasminogen and other proteins remains unclear (Refs. 33 and 34 and the present report), previous observation about the intrinsic sialyltransferase activity of insect cells does not contradict our results.

Because posttranslational glycosylation can influence the biological and pharmacokinetic properties of synthesized glycoproteins when they are used in vivo for clinical applications, researchers have attempted to modify baculovirus-insect cell systems to express glycoproteins with “mammalianized” N-glycans, particularly those with terminal sialylation. Recently, Jarvis and co-workers reported the occurrence of galactosylation and terminal sialylation on the baculoviral glycoprotein gp64 expressed by infection of SF9 cells with a bovine galactosyltransferase/rat sialyltransferase dual recombinant baculovirus (35) or by infection of TN5 cells expressing both enzyme genes with a wild-type baculovirus (36). Collectively, the insufficient synthesis of sialylated N-glycans by insect cells may depend on, at least in part, the intense activity of insect-specific GlcNAcases and the significant but low activities of galactosyltransferase and sialyltransferase. The biological significance of such insufficient synthesis remains to be elucidated. One possible explanation for this phenomenon is that it might help innate immune systems of insects. For example, insect lectins, which are major factors in host defense, recognize components of complex-type N-glycan such as GlcNAc and galactose (37, 38). Therefore, insect-specific N-glycosylation may contribute to self and non-self recognition upon microorganism infections.

In the present study, we demonstrated the expression of terminally sialylated recombinant proteins in insect cells after simply inhibiting their GlcNAcase activity and showed that this phenomenon was not protein-specific. Our system can be applied to various combinations of baculovirus-insect cells to produce recombinant glycoproteins with “mammalianized” N-glycans.

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