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INTRINSIC GLYCOSYLATION POTENTIALS OF INSECT CELL CULTURES AND INSECT LARVAE

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SUMMARY

The glycosylation and subsequent processing of native and recombinant glycoproteins expressed in established insect cell lines and insect larvae were compared. The *Spodoptera frugiperda* (Sf21) and *Trichoplusia ni* (TN-368 and BTI-Tn-5B1-4) cell lines possessed several intrinsic glycoproteins that are modified with both N- and O-linked oligosaccharides. The N-linked oligosaccharides were identified as both the simple (high mannose) and complex (containing sialic acid) types. Similarly, the *T. ni* larvae also possessed intrinsic glycoproteins that were modified with O-linked and simple and complex N-linked oligosaccharides. Additionally, human placental, secreted alkaline phosphatase (SEAP) produced during replication of a recombinant baculovirus in *T. ni* larvae was modified with complex oligosaccharide having sialic acid linked α(2–6) to galactose.

Key words: baculovirus; recombinant protein expression; glycosylation; insect cell culture; posttranslational processing.

INTRODUCTION

The baculovirus expression vector system (BEVS) in insect cell cultures has been used to express hundreds of foreign proteins (Luckow, 1991). These proteins are immunogenically, antigenically, and functionally similar to the authentic protein. Recently, expression of glycosylated proteins with this system has received increased attention. In particular, the integrity of the modifying oligosaccharide as compared to its authentic counterpart has been examined and debated.

It is known that the glycan moieties modifying a glycoprotein can have a significant influence on that protein's antigenicity, structural folding, solubility, and stability (Geisow, 1992). Varying the number and composition of the oligosaccharide moieties can alter the bioactivity for many glycoproteins. In particular, the elimination of the terminal sialic acid residues from complex-type oligosaccharides can dramatically reduce the in vivo bioactivity. For example, the removal of sialic acid from the proteins erythropoietin and tissue plasminogen activator resulted in the formation of biologically inactive proteins (Hotchkiss et al., 1988; Howard et al., 1991).

Previously, studies reported that vertebrate glycoproteins modified with complex oligosaccharide side chains have truncated, high mannose oligosaccharide side chains when produced with the BEVS in insect cells (Steiner et al., 1988; Jarvis et al., 1990; Kuroda et al., 1990; Vialard et al., 1990; Vissavajjhala and Ross, 1990; Wathen et al., 1991). Similar results were obtained by our laboratory in which the glycans modifying a secreted alkaline phosphatase protein (SEAP) expressed in six insect cell lines were evaluated and found to be only of the high mannose or simple oligosaccharide type (Davis et al., 1993).

However, higher processing of baculovirus-expressed glycoproteins has been reported with human plasminogen (HPg) and human chorionic gonadotropin expressed in *S. frugiperda* insect cells (Davidson et al., 1990; Sridhar et al., 1993). These studies demonstrated the presence of complex-type glycan-containing sialic acid. In subsequent experiments, Davidson and Castellino demonstrated that other insect cell lines, *Mamestra brassicae* (IZD-MB0503) and *Manduca sexta* (CM-1), also assembled complex oligosaccharide on HPg (1991). These observations were supported by reports describing the presence of two enzymes in uninfected insect cells that are essential in the conversion of simple to complex oligosaccharide (Davidson et al., 1991; Velardo et al., 1993). However, conflicting results recently were obtained by Kubelka et al. (1994) in a study evaluating the intrinsic N-linked glycosylation biosynthetic machinery of insect cells. Analysis of the glycan structures of membrane glycoproteins from Sf21, MB0503, and *Bombyx mori* (Bm-N) insect cells revealed the presence of only simple, oligomannose-type ($Man_2GlcNAc_2$ to $Man_9GlcNAc_2$) oligosaccharides.

In order to resolve these observed discrepancies in the glycan processing of insect cells, this study evaluated the influence that host factors have on protein glycosylation by investigating the differences in oligosaccharide composition of glycoproteins produced in the Sf21, BTI-Tn-5B1-4, and TN-368 insect cell lines. Furthermore, posttranslational processing events, such as proteolytic processing and amidation, have been reported to occur correctly and efficiently with recombinant proteins produced in insect larvae (Hellers et al., 1991; Gunne and Steiner, 1993). However, the N-linked glycosylation potential of recombinant proteins expressed in insect larvae has not been explored. In this study, we describe the N-linked oligosaccharides modifying SEAP expressed in *T. ni* larvae. The implications of both these studies' results on the expression of recombinant protein with the BEVS are discussed.

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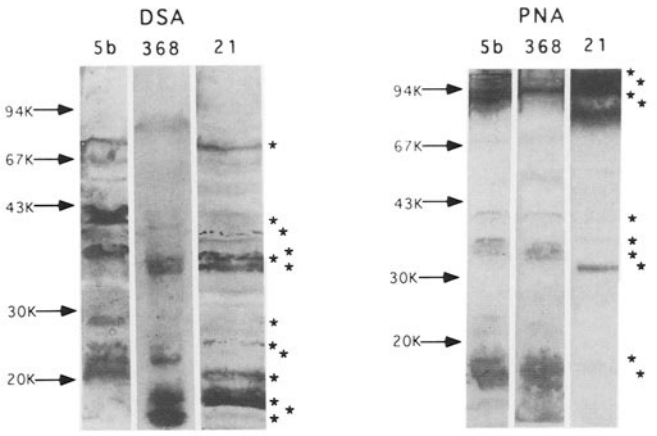

FIG. 1. Lectinoblot of homogenates from BTI-Tn-5B1-4 (5b), TN-368 (368), and Sf21 (21) cell cultures. Glycan differentiation was detected with *Datura stramonium* agglutinin (DSA) and *Peanut agglutinin* (PNA). * at right indicate intrinsic proteins that DSA or PNA bound to. *Arrows* at left are molecular weight markers (in daltons).

MATERIALS AND METHODS

Insects, cells, and viruses. The *Spodoptera frugiperda* (IPLB-SF21AE) (Sf21) (Passages #60–67) (Vaughn et al., 1977), *Trichoplusia ni* (BTI-Tn-5B1-4) (Passages #34–41) (Granados et al., 1994), and *T. ni* (TN-368) (Passages #86–89) (Hink, 1970) cells were grown in TNM-FH media, supplemented with yeastolate, lactalbumin hydrolysate, and 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT). The *T. ni* larvae were obtained from a laboratory colony maintained on high wheat germ diet (Bell et al., 1981), as described previously (Hughes et al., 1986).

The recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV) expressing SEAP (Ac-SEAP) was constructed as described previously (Davis et al., 1992).

Infection of insect larvae. The *T. ni* larvae were infected with the preoccluded form of Ac-SEAP (Wood et al., 1993). The preoccluded virus samples were prepared by infecting Sf21 cells in six-well plates (Falcon, Lincoln Park, NJ) (1×10^6 cells/well) at a multiplicity of infection of 10 plaque forming units per cell; 30 h postinfection, the cells were gently resuspended and centrifuged at 500 ×g for 5 min. The pellet was washed once with medium, resuspended in 1 ml of medium, sonicated, and stored at 4° C. Three microliter aliquots of the suspension were pipetted onto 3 mm cubes of high wheat germ diet that had been placed in 1-oz plastic cups. A 7-mm-square piece of water-saturated Whatman 3-mm filter paper (Clifton, NJ) was also placed in the cup to serve as a moisture reservoir to prevent desiccation of the diet cube. Individual late fourth instar *T. ni* larvae were allowed to feed on the contaminated food during incubation at 26° C. All larvae that had ingested the entire diet cube within 16 h were transferred to cups containing 2 ml of diet and incubated at 26° C.

Sample preparation, immunoprecipitation, and glycan detection. Cell culture samples were harvested and centrifuged in a microfuge (12 000 ×g) for 5 min. The pellet was washed once with phosphate buffered saline (PBS), pH 6.3, and centrifuged again. The pellet was resuspended and sonicated in a bath-type sonicator (Heat Systems-Ultrasonics, Plainview, NY). Immediately after sonication, the neuraminidase inhibitor, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Boehringer Mannheim Biochemicals, Indianapolis, IN), was added (1 m*M*), and the samples stored at −20° C (Warner et al., 1990). Protein levels for the three insect cell line homogenates were determined by the method of Bradford (1976). Aliquots containing equivalent amounts of total protein were vortexed with SDS-PAGE sample buffer, heated in a boiling water bath for 2 min, and subjected to SDS-PAGE according to Wood (1980).

Three days postfeeding, insect larvae were pooled, homogenized with a tissue homogenizer (Heat Systems-Ultrasonics), and centrifuged at 500 ×g for 10 min. The supernatant was collected, neuraminidase inhibitor added (1 m*M*), and samples stored at −20° C. Sample proteins were immunoprecipitated with a polyclonal antibody to placental alkaline phosphatase (Accurate Chemical and Scientific Corp., Westbury, NY), subjected to SDS-PAGE, and their glycan moieties detected using the method of Davis et al. (1993).

RESULTS AND DISCUSSION

Lectin analysis of cell culture homogenates. Homogenates from uninfected BTI-Tn-5B1-4, TN-368, and Sf21 cell cultures were fractionated by SDS-PAGE, transferred to PVDF membranes, and analyzed for glycan differentiation using lectins that specifically bind to various carbohydrate moieties (Figs. 1 and 2). The lectin *Datura stramonium* agglutinin (DSA), which specifically recognizes galactose (Gal)-linked β(1–4) to N-acetylglucosamine (GlcNAc), bound to several proteins of differing molecular weights from all three cell lines (Fig. 1). The DSA bound to BTI-Tn-5B1-4 cell line proteins with apparent molecular weights of 40 kilodaltons (kDa), 34 kDa, 25 kDa, and a group of lower molecular weight proteins (21–23 kDa). Similarly, the DSA bound to TN-368 cell line proteins with apparent molecular weights of 33 kDa, 21 kDa, 18 kDa, 17 kDa, and 15 kDa. The intrinsic proteins of Sf21 cells that DSA bound to had apparent molecular weights of 70 kDa, 37 kDa, 33 kDa, 32 kDa, 23 kDa, 18 kDa, and 17 kDa. These results indicate that insect cells possess the biochemical potential to process N-linked oligosaccharides to a complex-type glycan.

Peanut agglutinin (PNA), which recognizes the core disaccharide, Gal β(1–3)GlcNAc of O-glycosidically linked carbohydrates, bound to proteins with apparent molecular weights of 82–98 kDa, 41 kDa, 17 kDa, and 16 kDa for all three cell lines (Fig. 1). The BTI-Tn-5B1-4, TN-368, and Sf21 cells also had proteins at 36 kDa, 33 kDa, and 31 kDa, respectively, to which PNA bound. These results demonstrate that insect cell cultures possess the ability to modify intrinsic glycoproteins with O-linked oligosaccharides (Fig. 1). Recently, Licari et al. also determined that PNA bound to cellular proteins from *S. frugiperda* (Sf9), *T. ni* (TN-368), *Bombyx mori*, and *Malacosoma disstria* (MD108) cell lines (1993). Similar to this study's results, they also found that these proteins which PNA bound to had large apparent molecular weights.

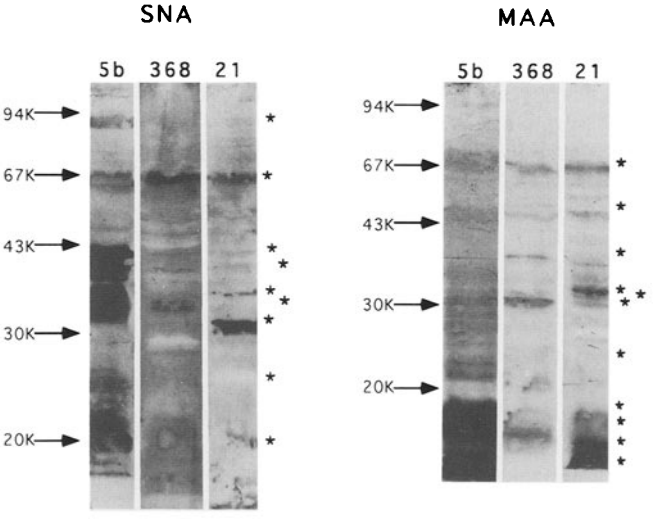

FIG. 2. Lectinoblot of homogenates from BTI-Tn-5B1-4 (5b), TN-368 (368), and Sf21 (21) cell cultures. Glycan differentiation was detected with *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA). * at right indicate intrinsic proteins that SNA or MAA bound to. *Arrows* at left are molecular weight markers (in daltons).

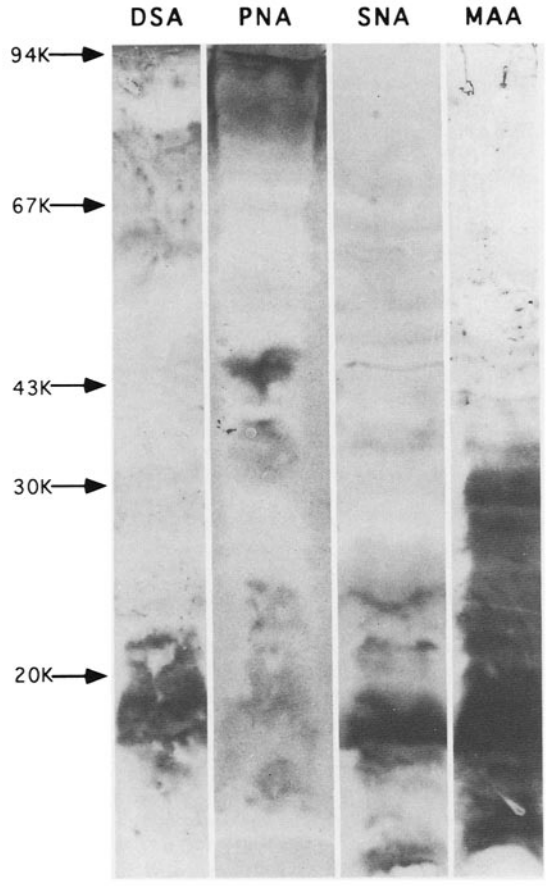

FIG. 3. Lectinoblot of uninfected *Trichoplusia ni* larvae. Glycan differentiation was detected with *Datura stramonium* agglutinin (DSA), *Peanut agglutinin* (PNA), *Sambucus nigra* agglutinin (SNA), and *Maackia amurensis* agglutinin (MAA). *Arrows* at left are molecular weight markers (in daltons).

The lectins *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) recognize sialic acid (SA) linked to Gal α(2–6) and α(2–3), respectively. SNA bound to several proteins from BTI-Tn-5B1-4 cell extracts having apparent molecular weights of 88 kDa, 67 kDa, 41 kDa, 40 kDa, 37 kDa, 26 kDa, and 20 kDa (Fig. 2). SNA bound to proteins from cell extracts of TN-368 cells with apparent molecular weights of 67 kDa and 37 kDa. Similarly, SNA bound to Sf21 cell extract proteins with apparent molecular weights of 67 kDa, 38 kDa, and 32 kDa. The proteins from all three cell lines that bound to MAA were lower in molecular weight (13–19 kDa) (Fig. 2). Many faint bands at various molecular weights were also observed for all three cell lines. These results demonstrate there were several intrinsic proteins from the cell lines, Sf21, BTI-Tn-5B1-4, and TN-368, that bind the lectins SNA and MAA (Fig. 2). This finding indicates that these proteins were modified with complex oligosaccharide-containing sialic acid. By contrast, Licari et al. (1993) did not detect significant binding of SNA to their tissue culture extracts, nor did Kubelka et al. (1994) observe any insect cell membrane glycoproteins modified with oligosaccharide-containing sialic acid. However, Licari et al. (1993) reported that cell lysates and cell-free supernatants from insect cell lines contain endogenous N-acetyl-β-glucosaminadase, N-acetyl-β-galactosaminidase, β-galactosidase, and sialidase activities. In the current study, the neuraminidase inhibitor, 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid, was added to prevent exoglycosidase activity.

Lectin analysis of uninfected/infected T. ni *larvae.* When samples of healthy *T. ni* larvae were analyzed for glycan differentiation, the homogenates were found to include proteins to which the four lectins DSA, PNA, SNA, and MAA bound (Fig. 3). DSA faintly bound to a protein with an apparent molecular weight of 19 kDa. The results for PNA were similar to those for the cell culture homogenates in that the proteins to which PNA bound had large apparent molecular weights (80–95 kDa). The lectin SNA bound to only one protein with an apparent molecular weight of 18 kDa, whereas MAA bound to several glycoproteins. These results support the conclusions of Roth et al. who recently described the presence of sialylated oligosaccharides throughout the development of *Drosophila melanogaster* (1992).

Since *T. ni* larvae appeared to possess the intrinsic biochemical machinery necessary to form complex oligosaccharides, *T. ni* larvae were infected with the Ac-SEAP virus, the hemolymph was immunoprecipitated with polyclonal antibody specific for placental alkaline phosphatase, fractionated by SDS-PAGE, and analyzed for glycan differentiation. There was no positive reaction with the lectin MAA, indicating there were no oligosaccharides with SA linked α(2–3) to Gal (Fig. 4). However, SNA bound to a protein with an apparent molecular weight of 64 kDa. This is the molecular weight reported for the mature form of SEAP (Davis et al., 1992). This suggests that SEAP contains terminal SA linked α(2–6) to Gal. It can also be seen in Fig. 4 that DSA bound to a protein with an apparent molecular weight of 34 kDa. The exact nature for this is unknown, but immunoprecipitated negative control samples (*T. ni* larvae infected with wild-type AcMNPV) did not yield similar results (data not shown). Furthermore, *Galanthus nivalis* agglutinin (GNA), a lectin that specifically recognizes terminal mannose linked α(1–3), α(1–6), or α(1–

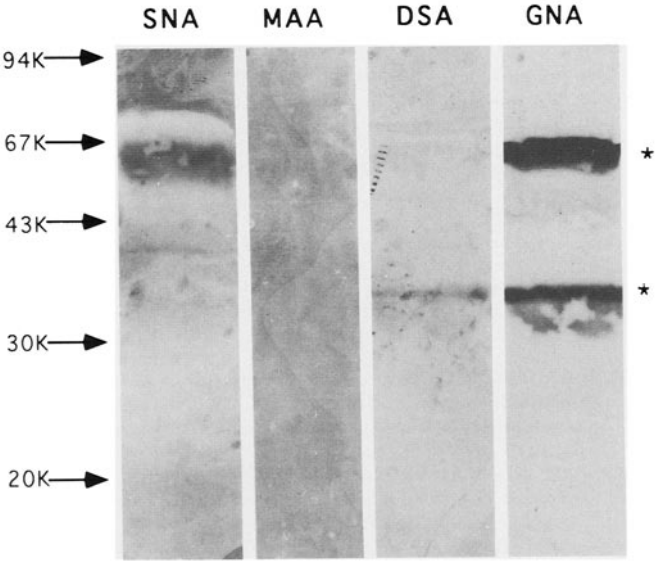

FIG. 4. Lectinoblot of immunoprecipitated proteins from Ac-SEAP infected *Trichoplusia ni* larvae. Glycan differentiation was detected with *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), *Datura stramonium* agglutinin (DSA), and *Galanthus nivalis* agglutinin (GNA). * at right indicate the position of the 64 and 34 kDa proteins. *Arrows* at left are molecular weight markers (in daltons).

2) to mannose, bound to both the 64 kDa and 34 kDa bands. Previously, when SEAP was expressed in six insect cell lines, it was found to be modified only with simple oligosaccharides (Davis et al., 1993). However, when SEAP was expressed in a Chinese hamster ovary cell line, the modifying oligosaccharide contained sialic acid. This unique result indicates that certain glycoproteins expressed with the BEVS in insect cell cultures may be modified with truncated, simple oligosaccharides, but when expressed in insect larvae are modified with more complex glycan structures. One hypothesis for this result may be that the larvae have a variety of different cell types, some of which allow for more heterogeneous glycan processing. From the large number and high concentration of intrinsic complex glycoproteins seen in this study, it is possible that complex oligosaccharide processing events are not reserved to a small number of cell types within the larvae.

This report supports previous investigations that have shown that insect cells possess the intrinsic biochemical processes to form complex-type N-linked oligosaccharides. However, the reason(s) for the discrepancy in glycan processing of recombinant glycoproteins expressed with the BEVS in cultured insect cells remains unknown. Although insect cells possess the appropriate pathways for complex oligosaccharide formation, only in two instances have recombinant glycoproteins been modified with sialylated glycans. This dichotomy may be the result of many differences between studies. For example, cell culture conditions, such as media and the infection processes, all differed. Also, physical structure of the expressed glycoprotein may influence the degree of glycan processing by a signal recognition mechanism, specific for insect cells, but different from mammalian cells. Continued studies evaluating these factors are necessary to determine the exact nature of each of these influences.

Furthermore, it was observed that the SEAP glycoprotein was modified with complex sialylated oligosaccharide when expressed in *T. ni* larvae. However, when SEAP was expressed in insect cell cultures derived from *T. ni* or *S. frugiperda*, it was modified with only simple-type glycans. This result indicates that expression of heterologous proteins in insect larvae may offer advantages in oligosaccharide processing. Further studies with other glycoproteins are necessary to determine if these observations are unique to this particular protein or whether they represent a more general event.

Acknowledgments

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