N-GLYCOSYLATION OF A BACULOVIRUS-EXPRESSED RECOMBINANT GLYCOPROTEIN IN THREE INSECT CELL LINES

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(Received 20 November 1996; accepted 6 March 1997)

SUMMARY

The capacity of two Trichoplusia ni (TN-368 and BTI-Tn-5b1-4) and a Spodoptera frugiperda (IPLB-SF-21A) cell lines to glycosylate recombinant, baculovirus-encoded, secreted, placental alkaline phosphatase was compared. The alkaline phosphatase from serum-containing, cell culture medium was purified by phosphate affinity column chromatography. The N-linked oligosaccharides were released from the purified protein with PNGase F and analyzed by fluorophore-assisted carbohydrate electrophoresis. The majority of oligosaccharide structures produced by the three cell lines contained two or three mannose residues, with and without core fucosylation, but there were structures containing up to seven mannose residues. The oligosaccharides that were qualitatively or quantitatively different between the cell lines were sequenced with glycosidase digestions. The S. frugiperda cells produced more fucosylated oligosaccharides than either of the T. ni cell lines. The smallest oligosaccharide produced by S. frugiperda cells was branched trimannose. In contrast, both T. ni cell lines produced predominantly dimannose and linear trimannose structures devoid of α 1-3-linked mannose.

Key words: Trichoplusia ni; Spodoptera frugiperda; insect cell culture; baculovirus; glycoprotein; alkaline phosphatase.

INTRODUCTION

The utility of the baculovirus expression system may be limited by the ability of insect cells to perform complex glycosylation of recombinant proteins. Considerable effort has been invested in baculovirus vector development (Luckow, 1991, 1995), but relatively little into investigation of the capacity of cultured insect cells to perform post-translational modifications. A few studies have compared the ability of different insect cell lines to glycosylate proteins (Davidson and Castellino, 1991a; Jarvis and Finn, 1995; Ogonah et al., 1996; Wagner et al., 1996). However, most glycoprotein studies have focused on the ability of an individual cell line to modify a particular recombinant protein. Accordingly, it is not clear to what extent the differences in glycosylation of most recombinant proteins result from intrinsic differences in the proteins being expressed or from differences in the cellular-processing potential.

Generally, recombinant glycoproteins produced in insect cell culture have only mannose attached to the core GlcNAc structure (reviewed in Luckow, 1991; Marz et al., 1995), but a few glycoproteins have been produced with glycans possessing terminal N-acetylglucosamine (GlcNAc) or sialic acid residues (Davidson et al., 1990; Davidson and Castellino, 1991a, 1991b; Sridhar et al., 1993; Jarvis and Finn, 1995; Ogonah et al., 1996; Wagner et al., 1996). Studies of the membrane glycoproteins from three insect cell lines (IPLB-SF-21AE, IZD-MB-0503, and Bm-N) revealed mostly oligosaccharides with terminal mannose structure, but a low percentage of oligosaccharides were terminated with GlcNAc residues in all three cell lines (Kubelka et al., 1994). Davis and Wood (1995) also found that insect cell (IPLB-SF-21AE, TN-368, and BTI-Tn-5b1-4) homogenates contain glycoproteins with terminal sialic acid. Some of the enzymes required to produce complex oligosaccharides on glycoproteins have been detected in insect cells (Altman et al., 1993), and their activities may be affected by baculovirus infection (Davidson and Castellino, 1991b; Davidson et al., 1991; Velardo et al., 1993; van Die et al., 1996). Some secreted glycoproteins isolated from the adult stages of insects also carry N-linked oligosaccharides with terminal sugars other than mannose (Staudacher et al., 1992a; Hård et al., 1993; Kubelka et al., 1993). Recombinant secreted placental alkaline phosphatase (SEAP) produced in Trichoplusia ni larvae shows evidence of complex glycosylation containing terminal sialic acid residues (Davis and Wood, 1995), but the quantitative importance of the sialoglycoconjugate proteins was not determined. The GP64 envelope glycoprotein of the budded form of the Autographa californica baculovirus contains mannose, fucose, and possibly GlcNAc when produced in three different infected insect cell lines (BTI-EAA, BTI-Tn-5b1-4, and IPLB-SF-21AE) but was sialylated only when expressed as a recombinant protein in a mammalian (COS-1) cell line (Jarvis and Finn, 1995). Based on the available evidence, it appears that most of the insect cell lines used for recombinant protein production have a reduced potential for complex glycosylation as compared to insect larvae and mammalian cell lines.

Because of the variation of glycosylation with different insect cell lines and insects, we have initiated a research program to identify cell lines that perform complex glycosylation with intrinsic and recombinant proteins. For these comparative studies, we are investigating the glycosylation of SEAP produced during replication of a
recombinant isolate of the *Autographa californica* nucleopolyhedrovirus (Davis et al., 1992, 1993; Davis and Wood, 1995). The SEAP is easily assayed, is efficiently secreted, and is processed to a complex glycoprotein (terminal sialic acid residues) when produced in mammalian cells (Takami et al., 1989; Chuang, 1989; Davis et al., 1995). Although SEAP has two putative glycosylation sites, only one is glycosylated (Millan, 1986). We describe here an affinity column purification procedure to purify SEAP from cell culture medium following synthesis in three insect cell lines commonly used with the baculovirus expression vector system. The SEAP-associated oligosaccharides were then fluorescently labeled and analyzed using fluorophore-assisted carbohydrate electrophoresis (FACE) procedures.

**Materials and Methods**

Cells and virus. The *T. ni* (TN-368) (Hink, 1970), *T. ni* (BFTi-Tr-5h1-4) (Granaresas et al., 1994), and *Spodoptera frugiperda* (IPLB-SF-21AE) (Vogelnest et al., 1977) cells were grown in TMM-FH media (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The recombinant *A. californica* nucleopolyhedrovirus expressing SEAP was constructed as previously described (Davis et al., 1992). Virus infections were performed according to Davis et al. (1995).

**Protein purification and analysis.** To purify SEAP, we modified a procedure that used 4-aminoazobenzophosphonic acid, diazotized to histidine, as an affinity support (Lindt et al., 1978). A phosphate affinity column was prepared by linking 4-aminoazobenzophosphonic acid to epoxy agarose via histidine. The histidine was first coupled to the epoxy agarose. Histidine monohydrochloride (HCl) and allowed to react for 20 min. This solution was added to the cold phosphate affinity matrix and eluted in a sintered-glass funnel with 1 M NaCl. M NaCl (pH 2.5) and then 0.2 M Tris, 0.5 M NaCl (pH 10.5) followed by washing with 2 l of deionized water.

The histidine-agarose was then diazotized to 4-aminoazobenzophosphonic acid. In an ice bath, 4.0 ml of 0.5 M sodium nitrite was added to 0.375 g of glycine. The reaction was stirred for 2 h in a fixed-angle rotor. The supernatant was concentrated two- to threefold by dialysis against polyethylene glycol (MW 15,000-20,000; Sigma Chemical Co., St. Louis, MO). The sample was then dialyzed against column buffer, 20 mM Tris, 1 M NaCl, (pH 8.0), and loaded on the phosphate affinity column. The column was washed with column buffer containing 1 M NaCl to remove unbound protein. The column was then washed with column buffer containing 1 M NaCl to remove unbound protein. The column was then washed with column buffer containing 1 M NaCl to remove unbound protein.

Protein purity was assessed by polyacrylamide gel electrophoresis (PAGE) and coomassie blue staining (Hames and Rickwood, 1981). The percent contaminating proteins was estimated from images generated with an image analysis system from Glyko (Novato, CA). Glycoproteins were detected on electrophoretic transmembrane transfers from PAGE (Towbin et al., 1979) to polyvinylidene difluoride (PVDF) membranes (DuPont, Boston, MA) (Gershoni and Palade, 1982, 1983). The glycopolypeptide detection was carried out with a digoxigenin glycan detection kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Western blot analysis (Towbin et al., 1979) was performed using similarly prepared PVDF membranes. A rabbit IgG from Accurate Chemical (Westbury, NY) was the primary antibody, and the secondary antibody was a goat, anti-rabbit, alkaline phosphatase conjugate (Sigma). SEAP enzyme activity assays were performed according to Davis et al. (1992).

Oligosaccharide preparation, labeling, and analysis. Oligosaccharides were released and labeling with 8-aminonaphthalene-1,3,6-trisulfonic acid were carried out with an N-linked oligosaccharide profiling kit from Glyko as described by Jackson (1994). The purified SEAP was concentrated and exchanged into a small volume of water with Centricon 30 concentrators from Amicon (Danvers, MA). Aliquots containing up to 50 micrograms of protein were lyophilized, denatured, and digested with PNGase F from Glyko at 37°C for 2 h or 25°C overnight. The oligosaccharides were labeled at 37°C overnight or 45°C for 3 h. The labeled oligosaccharides were fractionated by gel electrophoresis using Glyko N-linked oligosaccharide gels and Glyko's gel electrophoresis apparatus. Standards included a mixture of labeled glucose polymers and a mixture of two core oligosaccharides standards, Man[1-4]GlcNAc[1-4]GlcNAc and Man[1-4]GlcNAc[1-4]GlcNAc from Glyko. The electrophoretograms were analyzed with a Glyko FACE imaging system (CDG camera and image analysis software).

**Results**

**SEAP purification.** Established preparative purification methods for alkaline phosphatase use procedures that are based on gross structural characteristics (size or charge) of proteins or antibody affinity and could select for particular glycosylomorphs. To avoid this bias, an affinity purification method that used the catalytic activity of the enzyme was developed. A minimum of 80% of the SEAP activity in the culture supernatant bound to the affinity matrix and eluted in a single peak in the phosphate gradient. When the unbound SEAP was rechromatographed, a similar proportion was bound suggesting that the binding process did not exclude subpopulations. The protein from all the cell lines was over 98% pure based on quantitative analysis of images from coomassie blue-stained sodium dodecyl sulfate (SDS)-PAGE electrophoretograms. Protein transfers from PAGE gels detected a single, co-migrating band with the digoxigenin glycan detection and Western blot analyses.

**Oligosaccharide profiles.** In order to assess the heterogeneity of the N-linked SEAP oligosaccharides and to detect differences between the cell lines, the oligosaccharides were cleaved from the purified SEAP, fluorescently labeled, and separated by PAGE (Fig. 1). There were pronounced qualitative differences between the SEAP-associated oligosaccharides produced in the *T. ni* and *S. frugiperda* cell lines. The SEAP oligosaccharides from both *T. ni* lines had three low molecular weight bands that were not found in the *S. frugiperda* derived samples, bands 5b, 5c, and 6. Qualitative differences were also apparent, with bands 4b and 5a being the most striking. Note that band 3 was a single band in the SEAP oligosaccharides from