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Role of Glycosylation in the Transport of Recombinant Glycoproteins Through the Secretory Pathway of Lepidopteran Insect Cells

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Cell lines established from the Lepidopteran insect *Spodoptera frugiperda* (e.g., Sf9) are used routinely as hosts for the expression of foreign proteins by baculovirus vectors. Previously, we showed that human tissue plasminogen activator (t-PA) was expressed, N-glycosylated, and secreted by Sf9 cells infected with a recombinant baculovirus (Jarvis DL, Summers MD: Mol Cell Biol 9:214–223, 1989). We also showed that t-PA secretion was blocked by tunicamycin (TM), an inhibitor of N-glycosylation, but not by castanospermine (CS) or N-methyldeoxynojirimycin, inhibitors of the initial steps in N-linked oligosaccharide processing. This suggested that the addition, but not the processing, of N-linked oligosaccharides is required for the secretion of recombinant t-PA from baculovirus-infected Sf9 cells. In this study, we present a more generalized evaluation of the role of N-glycosylation in the transport of recombinant glycoproteins through the Sf9 cell secretory pathway. Several different secretory or membrane-bound glycoproteins were expressed in control, TM-treated, or CS-treated Sf9 cells, and their appearance in the medium or on the cell surface was measured. The results showed that TM blocked the transport of some, but not all, of these proteins, whereas CS did not block the transport of any. This suggests that N-glycosylation is sometimes required for the transport of recombinant glycoproteins through the Sf9 secretory pathway, while processing of the oligosaccharides is not. At least two other proteins, p80 and p31, consistently coimmunoprecipitated with the nonglycosylated precursors of recombinant glycoproteins expressed in TM-treated Sf9 cells. Neither was antigenically related to any of the recombinant proteins. Relatively larger amounts of p80 and p31 were coprecipitated when transport was completely blocked by TM compared to when transport was only reduced or was unaffected. These results suggest that p80 and p31 block the transport of some nonglycosylated glycoprotein precursors in TM-treated Sf9 cells by binding to them and producing transport-incompetent heterooligomeric complexes. If this speculation is correct, then p80 and p31 are functionally analogous to the mammalian immunoglobulin heavy chain binding/glucose-regulated 78 kilodalton protein (BiP/GRP78).

Key words: baculovirus expression vector, oligosaccharide processing, immunoglobulin heavy chain binding protein (BiP), glucose-regulated 78 kd protein (GRP78), tunicamycin, castanospermine

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Baculovirus expression vectors have become extremely important tools for the expression of foreign genes, both for basic research and for the production of proteins with direct clinical applications in human and veterinary medicine [1,2]. The laboratory hosts used for the expression of foreign proteins by recombinant baculoviruses are cell lines derived from Lepidopteran insects, which serve as the natural hosts for baculoviruses in the environment. A commonly used host is the Sf9 clone of IPLB-Sf21-AE, a cell line established from the fall armyworm, *Spodoptera frugiperda* [3]. With widespread and growing use of Lepidopteran insect cells for the production of foreign proteins by recombinant baculoviruses, interest in their basic biology, particularly in their protein processing pathways, has intensified.

In a previous study, we used a recombinant baculovirus to induce the expression and secretion of human tissue plasminogen activator (t-PA) in infected Sf9 cells as a model for protein processing studies [4]. Secretion of t-PA was blocked by treatment of the cells with tunicamycin (TM), which blocks the covalent addition of N-linked oligosaccharide side chains. However, t-PA secretion was unaffected by treatment with castanospermine (CS) or N-methyldeoxynojirimycin, which block the glucosidases involved in the initial processing of N-linked oligosaccharide side chains. The processing inhibitors induced an increase in the molecular weight of t-PA, indicating their ability to block insect cell glucosidase activities. These results suggested that N-glycosylation per se, but not oligosaccharide processing, is required for the secretion of recombinant t-PA from baculovirus-infected Sf9 cells. These results posed two major questions for further study. What are the roles of N-glycosylation and oligosaccharide processing in the movement of recombinant glycoproteins, in general, through the Sf9 cell secretory pathway? What molecular mechanism explains how the secretion of some proteins is blocked in TM-treated Sf9 cells?

In this study, we have addressed both these questions by surveying the effects of TM or CS treatment on the secretion or cell surface expression of several different recombinant glycoproteins in baculovirus-infected Sf9 cells. The results show that N-glycosylation is required for the transport of some, but not all, foreign glycoproteins through the Sf9 cell secretory pathway, whereas oligosaccharide processing generally appears to be dispensable. The results also show that the nonglycosylated precursors of several different recombinant glycoproteins are associated with at least two other proteins in TM-treated Sf9 cells, designated p80 and p31. These proteins are not antigenically related to any of the recombinant products, and their presence correlates inversely with transport through the secretory pathway. We propose that a physical interaction between p80, p31, and a nonglycosylated recombinant glycoprotein precursor in TM-treated Sf9 cells produces a transport-incompetent heterooligomeric complex. If this speculation is correct, then p80 and/or p31 are Sf9 cell analogs of the mammalian immunoglobulin heavy chain binding/glucose-regulated 78 kilodalton protein (BiP/GRP78), for which this activity has been proposed previously [5–8; reviewed in 9, 10].

MATERIALS AND METHODS

Cells and Viruses

The methods used for the culture of Sf9 cells and wild-type baculovirus and for the construction, purification, and propagation of recombinant baculoviruses have been described in a manual written for users of the baculovirus expression vector system [11]. The baculoviruses used in this study included strain E2 of wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and several recombinant derivatives,

including viruses that express human β-interferon (941-IFN; D.L. Jarvis, unpublished; similar to Ac-380-IFN-β in Smith et al. [12]), human t-PA (Ac373-TPA [13]), the Sindbis virus 26S transcription unit (Ac373-SV26 [14]), and the Simian virus 40 (SV40) large tumor antigen (373-T [15]). BHK cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum.

Infections, Labeling, and Extractions

Sf9 cells were infected as described previously [11], and TM or CS treatments were initiated at various times postinfection. During the latter part of the treatment periods, control or treated cells were pulse-labeled with [^{35}S]Translabel (ICN Biomedicals Inc.) in methionine-free Grace's medium; then extracellular, intracellular, and insoluble fractions were prepared as described previously [4,14]. Briefly, the extracellular fraction was the clarified labeling medium, adjusted to 1.0% nonidet P40 (NP40), the intracellular fraction was a clarified 1.0% NP40 cellular extract, and the insoluble fraction was the NP40-insoluble residual boiled for 5 min in protein sample buffer (50 mM Tris HCl, pH 6.8; 4% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol, 1 mg/ml bromphenol blue). Extracts were prepared for western blotting analysis by removing the medium from unlabeled cells and treating them with protein sample buffer. Cell surface labeling was performed at 24 hr postinfection (hpi), using the lactoperoxidase-catalyzed radioiodination method as described previously [16]. The precise timing, labeling conditions, and inhibitor treatment conditions used in each experiment are specified in the figure legends.

Radioimmunoprecipitation and Western Blotting Analysis

Radioimmunoprecipitations and discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously [4,14,17]. Western blotting was performed using modifications of the methods originally described by Towbin and coworkers [18] as described previously [4,14]. Immune complexes were detected in western blots by using alkaline phosphatase-conjugated second antibodies as described previously [19].

RESULTS AND DISCUSSION

Role of N-Glycosylation in Secretion

The role of N-glycosylation in the secretion of recombinant t-PA from Ac373-TPA-infected Sf9 cells was evaluated in previous experiments by pulse-labeling control or inhibitor-treated cells and testing the extracellular, intracellular, and insoluble fractions for t-PA-related proteins by radioimmunoprecipitation or western blotting [4]. t-PA was detected in the extracellular fraction of untreated cells and a slightly larger form was detected in the extracellular fraction of CS-treated cells. However, t-PA was not secreted from TM-treated cells. Moreover, significant amounts of a nonglycosylated t-PA precursor accumulated in the insoluble fraction of TM-treated cells, whereas the control or CS-treated cells contained little t-PA in this fraction. These findings suggested that N-glycosylation, but not oligosaccharide processing, is required for the secretion of human t-PA from baculovirus-infected Sf9 cells and that an alteration in the intracellular distribution of t-PA occurs in the absence of N-glycosylation.

Analogous experiments were performed to determine if N-glycosylation or oligosaccharide processing are required for the secretion of another human glycoprotein,

β-interferon (Fig. 1). The intracellular fraction of 941-IFN-infected Sf9 cells contained three major forms of this protein (marked by the closed circles in Fig. 1). The largest of these is N-glycosylated; it was not detected in TM-treated cells (Fig. 1) and it disappeared after treatment with peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidase (PNGase; data not shown), an enzyme that removes N-linked oligosaccharide side chains [20]. The other two are nonglycosylated forms of β-interferon; neither was affected by TM or PNGase. The extracellular fraction of 941-IFN-infected Sf9 cells contained two forms of β-interferon (marked by the open circles in Fig. 1). The larger protein is N-glycosylated, as shown by its absence in TM-treated cells (Fig. 1) and its sensitivity to PNGase (data not shown). The smaller is nonglycosylated, as shown by its resistance to both reagents. The key observation is that the smallest form of interferon, which is nonglycosylated, was detected in the extracellular fraction of both control and TM-treated cells (Fig. 1). The ability of baculovirus-infected Sf9 cells to secrete this nonglycosylated form of β-interferon indicates that N-glycosylation is not a universal requirement for the secretion of all foreign proteins from these cells. Furthermore, the ability of TM-treated Sf9 cells to secrete this nonglycosylated protein shows that the

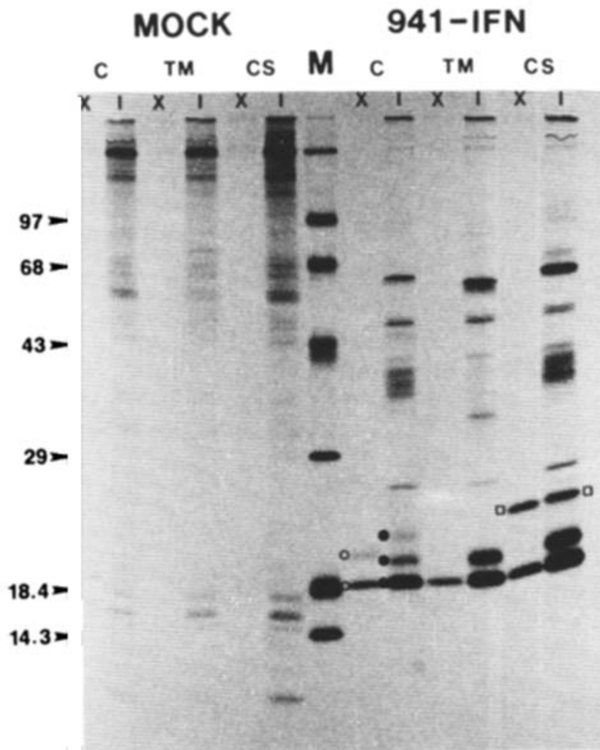


Fig. 1. Effects of TM or CS on the expression and secretion of human β-interferon. Sf9 cells were mock-infected or infected with 941-IFN, then treated with medium alone (C), with 1.0 μg/ml TM, or with 200 μM CS from 1 to 24 hpi. The cells were pulse-labeled with 100 μCi/ml [^{35}S]Translabel from 20 to 24 hpi; then extracellular (X) and intracellular (I) fractions were prepared and treated with polyclonal rabbit anti-human β-interferon. The immunoprecipitates were harvested, washed, and analyzed by discontinuous SDS-PAGE on a 15% polyacrylamide gel. The specifically immunoprecipitated interferon polypeptides are marked with open circles, closed circles, or squares, as indicated in the text. The sizes of the molecular weight markers (M) are indicated in thousands by the numbers on the side of this and subsequent autoradiograms.

previously observed inhibition of t-PA secretion was a direct or indirect consequence of preventing the N-glycosylation of t-PA; it was not a nonspecific effect of TM treatment on Sf9 cells.

Yet another form of β-interferon was detected in both the intracellular and the extracellular fractions of CS-treated Sf9 cells (marked by the square symbols in Fig. 1). It was sensitive to PNGase treatment and was larger than any of the other N-glycosylated forms, suggesting that it contains unprocessed N-linked oligosaccharide side chains. Thus, as for t-PA, processing of the carbohydrate side chains does not appear to be required for the secretion of β-interferon from Sf9 cells. The difference in the sizes of the glycosylated forms of β-interferon detected in the extracellular and in the intracellular fractions of untreated cells suggests that the oligosaccharide side chains of β-interferon are processed during transport through the Sf9 cell secretory pathway. The elimination of this size difference by CS treatment supports this interpretation. The ability of insect cells, in general, to trim N-linked oligosaccharides to smaller forms has been documented in previous studies by other investigators [21–23] and was documented specifically for Sf9 cells in our previous study of recombinant t-PA processing [4].

Role of N-Glycosylation in Cell Surface Expression

Additional experiments were performed to evaluate the role of N-glycosylation in the cell surface expression of foreign membrane-bound glycoproteins in Sf9 cells. These represent a second class of glycoproteins that are transported through the cellular secretory pathway. The methodology was slightly different; instead of pulse-labeling the infected cells, as in the t-PA and β-interferon studies, the cells were radioiodinated by a method that labels only proteins exposed on the cell surface [16]. Using this method, we observed nonspecific immunoprecipitation of several proteins from mock-infected cells (marked by the square symbols in Fig. 2). Fortunately, these proteins were detected only in relatively minor amounts in cells infected with wild-type or recombinant baculoviruses. Thus it was possible to see that the envelope glycoproteins, gP64 of AcMNPV (marked by the closed circles in Fig. 2), and pE62, E1, and E2 of Sindbis virus (in order of decreasing size; marked by the open circles in Fig. 2A [14]) were expressed on the surface of infected Sf9 cells. In TM-treated infected cells, a nonglycosylated gP64 precursor was detected on the cell surface (closed circles), but nonglycosylated Sindbis virus glycoprotein precursors were not (Fig. 2A). CS treatment induced a small increase in the size of gP64, reflecting inhibition of oligosaccharide trimming, but it did not prevent the expression of the immature glycoprotein on the cell surface (Fig. 2B). At this time, the effect of CS treatment on the expression and processing of the Sindbis virus glycoproteins has not been evaluated.

Together with the results of the t-PA and β-interferon studies, these results show that N-glycosylation, but not oligosaccharide processing, is required for the transport of some, but not all, secretory or membrane-bound glycoproteins through the secretory pathway of Sf9 cells. In this respect, these Lepidopteran insect cells are identical to mammalian cells, for which the same general conclusion is valid [24].

Association of p80 and p31 With Nonglycosylated Glycoprotein Precursors

In the original studies on the effects of TM on t-PA secretion, we noticed that several proteins reproducibly coimmunoprecipitated with the nonglycosylated t-PA

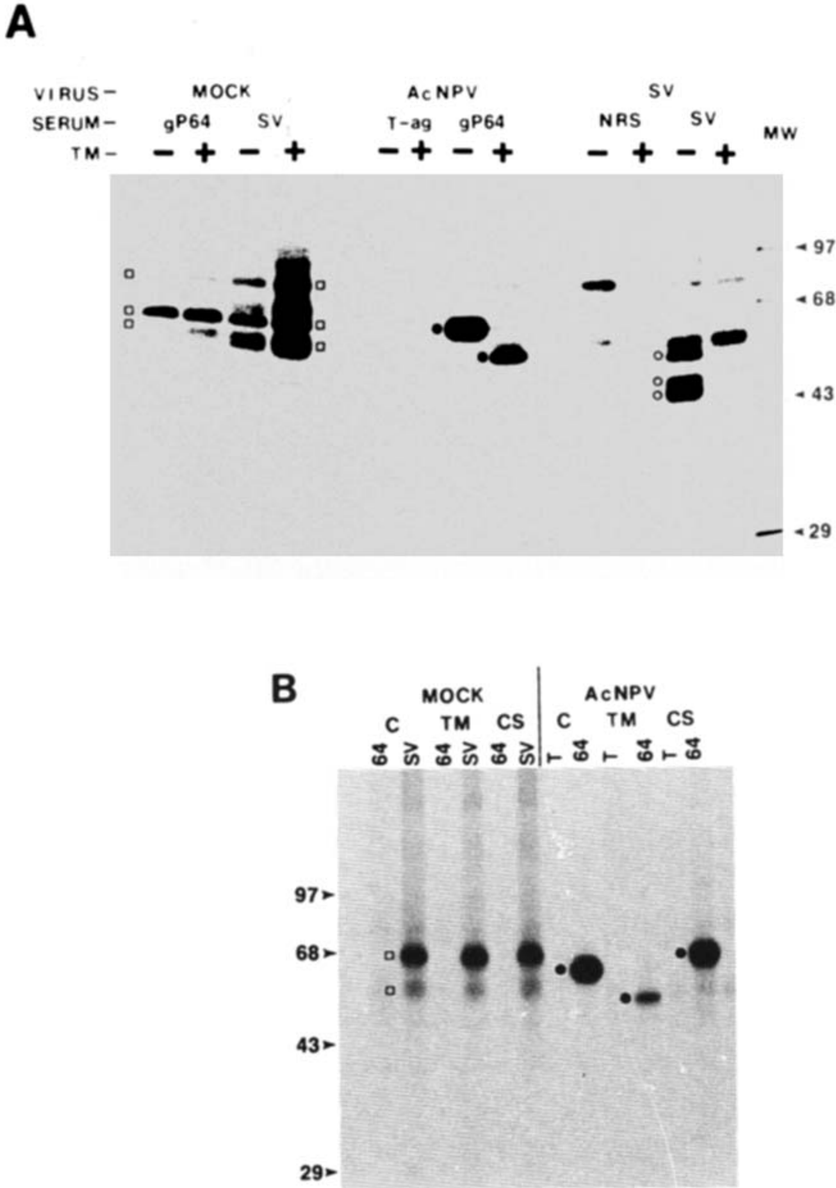


Fig. 2. Effects of TM and CS on cell surface expression of various recombinant proteins. Sf9 cells were mock-infected or were infected with AcMNPV or Ac373-SV. The cells were treated with medium alone, 1.0 μg/ml TM, or 200 μM CS from 1 to 24 hpi; then they were surface radioiodinated. Intracellular fractions were prepared and treated with monoclonal antibodies against AcMNPV gP64 (64) or SV40 large tumor antigen (T), with polyclonal antisera against purified Sindbis virus (SV), or with normal rabbit serum (N). Immunoprecipitates were harvested, washed, and analyzed by SDS-PAGE on 12% polyacrylamide gels. **A** shows control (–) or TM-treated (+) cells. **B** shows control (C), TM-treated (TM), and CS-treated (CS) cells. Nonspecifically precipitated bands are marked by squares, gP64-related bands are marked by closed circles, and Sindbis virus glycoproteins are marked by open circles.

precursor from TM-treated Sf9 cells [4]. None of these reacted with anti-t-PA in western analyses, indicating that they were precipitated nonspecifically. This led us to speculate that, in TM-treated Sf9 cells, the secretion of t-PA might be blocked by a physical association between the nonglycosylated t-PA precursor and other, unrelated proteins. We also speculated that this mechanism might exist by design; i.e., Sf9 cells might contain cellular proteins which bind to immature or aberrant protein precursors in the rough endoplasmic reticulum to prevent their secretion, by analogy to one of the functions proposed for the mammalian GRP78/BiP protein [5–8; reviewed in 9, 10]. To evaluate this possibility further, we performed radioimmunoprecipitation and SDS-PAGE analysis with extracts from control or TM-treated Sf9 cells expressing gP64, t-PA, the Sindbis virus structural proteins, or the SV40 large tumor antigen (T-ag; Fig. 3). SV40 T-ag, which is a glycoprotein that does not enter the cellular secretory pathway [25–29], was included as a negative control. Examination of the SDS-PAGE profiles revealed that at least two proteins, with apparent molecular weights of about 80,000 (p80) and 31,000 (p31), were consistently coimmunoprecipitated with the nonglycosylated precursors of foreign glycoproteins from TM-treated cells (marked by stars in Fig. 3). These proteins were not coprecipitated with the foreign glycoproteins from untreated cells, nor were they precipitated from TM-treated cells expressing SV40 T-ag.

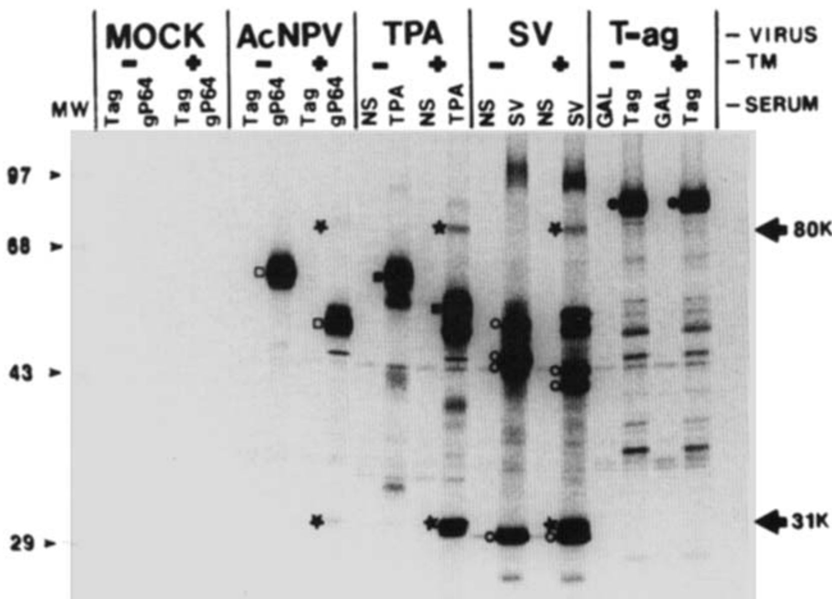


Fig. 3. Coimmunoprecipitation of p80 and p31 with nonglycosylated recombinant glycoprotein precursors. Sf9 cells were mock-infected or infected with AcMNPV, Ac373-TPA, Ac373-SV, or 373-T. The cells were treated with medium alone (–) or with 2.0 μg/ml TM (+) from 28 to 36 hpi; then they were pulse-labeled with 200 μCi/ml of [^{35}S]Translabel for 15 min. After labeling, intracellular fractions were prepared and treated with monoclonal antibodies against SV40 T-ag (Tag), AcMNPV gP64 (gP64), or *Escherichia coli* β-galactosidase (GAL); with polyclonal goat anti-t-PA (TPA) or rabbit anti-Sindbis virus (SV); or with normal goat or rabbit serum (NS). Immunoprecipitates were harvested, washed, and analyzed by SDS-PAGE on a 12% polyacrylamide gel. The open boxes mark the positions of gp64 and its precursor. The closed boxes mark the positions of t-PA and its precursor. The open circles mark the positions of the Sindbis virus envelope glycoproteins and their precursors as well as the nonglycosylated capsid protein (29K). The closed circles mark the position of SV40 T-ag. The stars mark the positions of p80 and p31.

There was an inverse relationship between the amounts of p80 and p31 coprecipitated and the amount of a particular nonglycosylated precursor transported; for example, transport of the gP64 precursor to the cell surface was inhibited, but not completely blocked, and relatively less p80 and p31 were coprecipitated. Neither p80 nor p31 was detectably coprecipitated with the nonglycosylated β-interferon precursor, which was secreted at normal levels from TM-treated cells (Fig. 1). In another experiment, p80 and p31 were shown to coimmunoprecipitate with nonglycosylated t-PA under a variety of different TM treatment conditions, and the presence of these proteins always correlated with the presence of the nonglycosylated precursor (Fig. 4). Finally, western blotting analyses were performed to determine if any of the antisera used in this study would react specifically with p80 or p31 (Fig. 5). None of the antisera reacted with proteins in the 80,000 or 30,000 molecular weight regions, indicating that p80 and p31 were, in fact, precipitated by a nonspecific mechanism. Together, these observations strongly suggest that a physical association with other, unrelated proteins, including p80 and p31, blocks the secretion of some glycoprotein precursors from TM-treated Sf9 cells. This interaction might, in turn, mediate a tight interaction with cytoskeletal components, which would account for the substantial amounts of nonglycosylated t-PA found in the NP40-insoluble fraction of TM-treated Sf9 cells [4].

Relationship Between p31, p80, and Mammalian BiP/GRP78

Because the function proposed for p80 and p31 is analogous to that proposed previously for the highly conserved mammalian protein, BiP/GRP78 [5–8; reviewed in 9, 10], we have tested a monoclonal antibody against mouse BiP/GRP78 [6] for

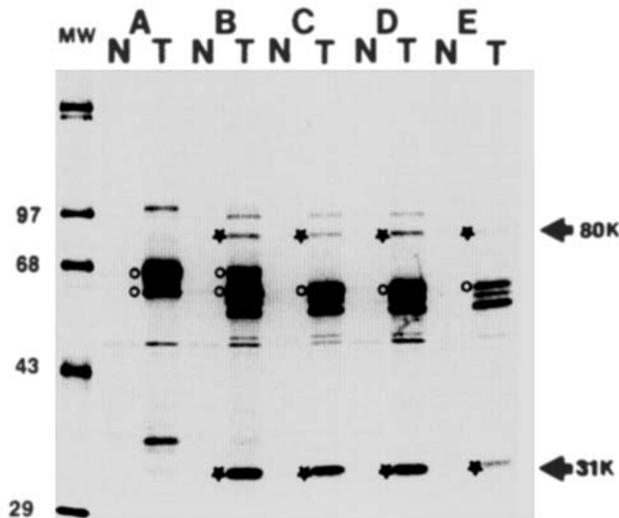
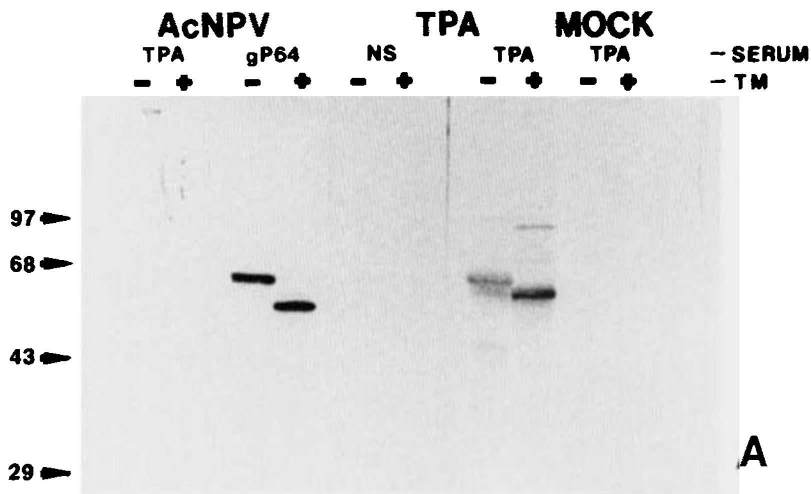
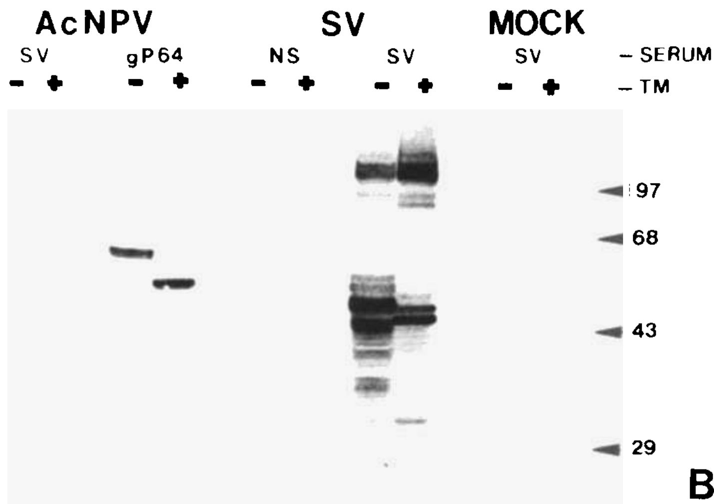


Fig. 4. Detection of p80 and p31 under various TM treatment conditions. 373-TPA-infected Sf9 cells were treated with medium alone (**A**), with 1.0 μg/ml TM for 2 hr (**B**) or 8 hr (**C**) or with 10.0 μg/ml TM for 2 hr (**D**) or 8 hr (**E**). TM treatments were from 16 to 24 hpi or from 22 to 24 hpi, and the cells were pulse-labeled with 200 μCi/ml [^{35}S]Translabel for 15 min at 24 hpi. After labeling, intracellular fractions were prepared and treated with normal goat serum (N) or goat anti-t-PA (T), and immunoprecipitates were harvested, washed, and analyzed by SDS-PAGE on a 12% polyacrylamide gel. Open circles mark the positions of glycosylated and nonglycosylated t-PA bands; stars mark the positions of the associated proteins, p80 and p31.





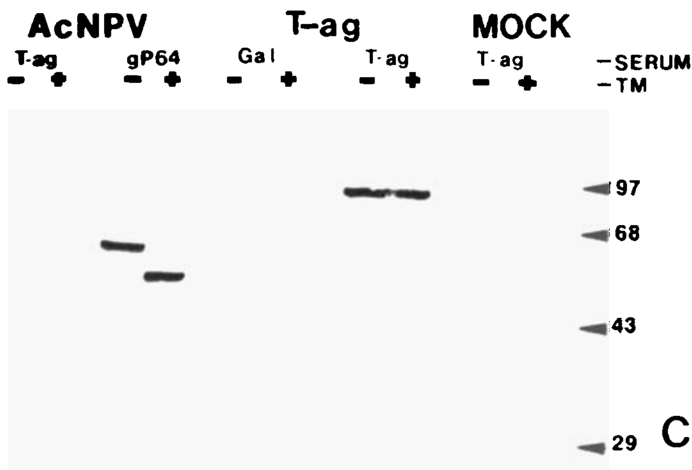


Fig. 5 A–C

reactivity with p80 and p31 (data not shown). A positive control experiment showed clearly that the antibody precipitated a BHK cell protein of about 75,000 in apparent molecular weight, the expression of which was elevated in TM-treated cells. However, neither p80 nor p31 was precipitated with this antibody. Obviously, this result must be interpreted cautiously; it shows only that neither p80 nor p31 is structurally homologous to mouse BiP/GRP78. We cannot eliminate the possibility that p80 or p31 is structurally related to BiP/GRP78 but lacks the single epitope recognized by the monoclonal antibody. At this time, we do not have a polyclonal antiserum against mammalian BiP/GRP78 or against the insect cell p80 or p31, which would permit a much more definitive test of their possible structural relationships. We intend to pursue this approach in the future. In addition, we plan to test the potential relationship between BiP/GRP78, p80, and p31 at the genetic level, by using a hamster GRP78 cDNA probe [30] generously provided to us by Dr. Amy Lee. Total cytoplasmic RNA from uninfected or infected, control or TM-treated Sf9 cells will be tested for the presence of GRP78-related sequences by dot-blot hybridization. This will permit a better evaluation of the possibility that p80 and/or p31 are related to mammalian GRP78, and will reveal whether they are virus- or Sf9 cell-encoded products.

SUMMARY

This study has shown that TM treatment inhibits the secretion or cell surface expression of some, but not all, recombinant glycoproteins in Sf9 cells. CS treatment did not inhibit the secretion or cell surface expression of any of the glycoproteins tested. These results suggest that N-glycosylation per se, but not oligosaccharide processing, is required for the transport of some, but not all, recombinant proteins through the Sf9 cell secretory pathway. The ability of Sf9 cells to secrete a nonglycosylated form of β-interferon suggested that the inhibition of secretion by TM, when it occurs, is a direct or indirect effect of preventing N-glycosylation of the recombinant protein; it is not simply a nonspecific effect of the TM treatment on Sf9 cells. The inhibition of secretion or cell surface expression by TM treatment is accompanied by an association between the nonglycosylated precursors of different recombinant glycoproteins with at least two unrelated proteins, p80 and p31. There is a strong correlation between the inhibition of transport by TM treatment and the association with these proteins. We propose that transport is inhibited by the association of a nonglycosylated precursor with p80 and p31, which results in the formation of transport-incompetent heterooligomeric complexes. This activity has been proposed previously for the mammalian protein BiP/GRP78 [5–8; reviewed in 9, 10]. Although a preliminary attempt to show a structural relationship between p80 or p31 and a mammalian BiP/GRP78 protein was unsuccessful, this experiment was not conclusive. In the future, we will perform additional experiments to test the possibility that p80 and p31 are structurally and/or genetically related to mammalian BiP/GRP78.

Fig. 5. Western blotting analysis of p80 and p31. Mock-infected, AcMNPV-infected, Ac373-t-PA-infected, Ac373-SV-infected, or 373-T-infected Sf9 cells were treated with medium alone (−) or with 2.0 μg/ml TM (+) from 12 to 36 hpi. The cells then were extracted, extracts were separated by SDS-PAGE on 12% polyacrylamide gels, and the proteins were blotted to nitrocellulose sheets. The sheets were blocked and probed with the indicated antibodies. Immune complexes were detected by using an alkaline phosphatase method as described in Materials and Methods. AcNPV-infected and mock-infected control extracts are compared to Ac373-t-PA-infected extracts in **panel A,** to Ac373-SV-infected extracts in **panel B,** and to 373-T-infected extracts in **panel C.**

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