Biosynthesis and processing of Spodoptera frugiperda α-mannosidase III

Brian R. Francis3, Laura Paquin3, Carla Weinkauf1,3, and Donald L. Jarvis2,3

1Department of Molecular Biology, University of Wyoming, P.O. Box 3944, Laramie, WY 82071-3944, USA
2To whom correspondence should be addressed; E-mail: dljarvis@uwyo.edu
3Present affiliation: Ambion, Inc., Austin, TX

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We previously cloned a lepidopteran insect cell cDNA that encodes a class II α-mannosidase that is localized in the Golgi apparatus but is cobalt-dependent, has a neutral pH optimum, hydrolyzes Man9GlcNAc2 to Man5GlcNAc2, and cannot hydrolyze GlcNAcMan9GlcNAc2. This enzyme was designated SfManIII to distinguish it from Golgi α-mannosidase II and indicate its derivation from the fall armyworm Spodoptera frugiperda. In the present study, we prepared a polyclonal antibody and used it to study the biosynthesis and processing of SfManIII. The results showed that Sf9 cells produce at least three different forms of SfManIII. SfManIII is initially synthesized as a precursor glycoprotein, which is slowly converted to two smaller end products with at least some endolysosomal H-resistant N-glycans. The smallest form of SfManIII is the only one of these two products that accumulates in the extracellular fraction. Tunicamycin blocked the production of SfManIII activity and the secretion of SfManIII protein and activity. Castanospermine blocked production of the large SfManIII product, retarded production of the smaller, increased intracellular SfManIII activity, and decreased extracellular SfManIII activity. Together, these results indicate that SfManIII is initially synthesized as a high-mannose glycoprotein precursor, its N-glycans are trimmed as it is transported to the Golgi apparatus, and a subpopulation, which appears to be proteolytically cleaved, is secreted in enzymatically active form. N-glycosylation is required for the production of active SfManIII, and N-glycosylation and N-glycan trimming are both required for the efficient secretion of an active form of this protein.

Key words: glycoproteins/insect/mannosidase

Introduction

It is well known that protein N-glycosylation, which involves the addition of an oligosaccharide side chain to a polypeptide via an amide linkage, is accomplished by an elaborate pathway in eukaryotic cells (Kornfeld and Kornfeld, 1985; Varki et al., 1999). A precursor glycan, Glc3Man9GlcNAc2, is initially transferred to newly synthesized proteins in the lumen of the endoplasmic reticulum (ER). Subsequently, this precursor is converted to various mature forms by enzymes localized along the cellular secretory pathway. Processing begins with the removal of three glucose residues by ER α-glucosidases, which produce Man5GlcNAc2, a “high-mannose” N-glycan. In higher eukaryotes, high-mannose N-glycans may be further processed by ER and Golgi α-mannosidases, which remove up to six mannose residues. Removal of these mannose residues is required for the subsequent conversion of N-glycans to “hybrid” or “complex” forms by various glycosyltransferases.

The α-mannosidases involved in N-glycan processing fall into two distinct classes with different substrate specificities, intracellular locations, sizes, cation requirements, sensitivities to plant alkaloid inhibitors, and amino acid sequences (reviewed in Moremen et al., 1994; Herscovic, 1999; Moremen, 2000). There are several class I α-mannosidases in the ER and Golgi complex, which function to remove the four α1,2-linked mannose residues from Man5GlcNAc2 and convert this substrate to ManGlcNAc2. A well-known class II enzyme, Golgi α-mannosidase II, is also involved in N-glycan processing. The substrate for this enzyme is produced by N-acetylglucosaminyltransferase I, which converts Man5GlcNAc2 to GlcNAcMan5GlcNAc2. Golgi α-mannosidase II cleaves the terminal α1,3- and α1,6-linked mannose residues from this substrate to produce GlcNAcMan5GlcNAc2. However, it has long been suspected that eukaryotic cells have other processing class II α-mannosidases as well (Fukuda et al., 1990). One is α-mannosidase II′, which is a Golgi enzyme that can convert Man5GlcNAc2 to Man4GlcNAc2 (Misago et al., 1995; Oh-Eda et al., 2001). Another is α-mannosidase III, which can convert Man5GlcNAc2 to Man4GlcNAc2 and compensate, at least in part, for the absence of Golgi α-mannosidase II in null mice (Chui et al., 1997).

In a previous study, our group cloned and characterized a cDNA encoding a class II α-mannosidase from the lepidopteran insect cell line, Sf9 (Jarvis et al., 1997). The deduced amino acid sequence of this enzyme was similar to those of various mammalian Golgi α-mannosidase II′s and, like the latter enzymes, the Sf9 α-mannosidase is an integral membrane glycoprotein with type II topology that hydrolyzes p-nitrophenyl-α-(D)-mannopyranoside (pNP-α-Man) and is inhibited by swainsonine (Jarvis et al., 1997). We subsequently demonstrated that this Sf9 class II α-mannosidase is localized in the Golgi apparatus. But we also found that this enzyme is distinct from Golgi α-mannosidase II′, because it is activated by cobalt and it can hydrolyze oligosaccharides containing terminal mannose residues but not GlcNAcMan5GlcNAc2 (Kawar et al., 2001). Based on these properties, we designated this insect cell enzyme Sf9 α-mannosidase III (SfManIII) and...
speculated that it might function in N-glycan processing in Sf9 cells. In the present study, we produced a rabbit antiserum against SfManIII, used it to examine the biosynthesis and processing of this protein, and examined the role of N-glycosylation and N-glycan trimming in the production of SfManIII protein and activity. The results of this study provide the first information available on the biosynthesis and processing and influence of N-glycosylation on the behavior of an α-mannosidase III.

Results

Production of a polyclonal, monospecific rabbit antiserum against SfManIII

A recombinant baculovirus was used to express the full-length, untagged SfManIII protein under the control of the polyhedrin promoter, as described previously (Jarvis et al., 1997). Sf9 cells infected with this virus produced large amounts of a protein that migrated as a broad band with an apparent molecular weight around 130 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; data not shown; see Figure 8 in Jarvis et al., 1997). This protein was identified as SfManIII based on its size, abundance, and absence in mock- or wild-type baculovirus-infected Sf9 cells. Thus, it was partially purified by preparative SDS–PAGE and used as the immunogen for the production of a polyclonal, monospecific rabbit antiserum, as described in Materials and methods. The resulting antiserum was shown to be specific for SfManIII in both radioimmunoprecipitation and immunoblotting assays, as described.

Biosynthesis of SfManIII

Radioimmunoprecipitation assays revealed that the new antiserum precipitated no major proteins of the expected size from mock- or wild-type baculovirus-infected Sf9 cell lysates (Figure 1A). Immunoblotting assays of wild-type baculovirus-infected Sf9 cell lysates provided the same results (Figure 1B). Thus the endogenous levels of SfManIII in uninfected and wild-type baculovirus-infected Sf9 cells are below the detection limits of these assays. Accordingly, it was necessary to express the SfManIII protein at higher levels by infecting Sf9 cells with a recombinant baculovirus expression vector. For the purposes of this study, it was important to circumvent potential limitations on protein processing that might be associated with massive overproduction of the protein during the late stages of infection with a conventional baculovirus expression vector (Jarvis, 1997). Therefore, we used an “immediate early” baculovirus expression vector, AcP(+)IESManIII, in which transcription of the SfManIII cDNA is controlled by a baculovirus immediate early (ie1) promoter. It is established that the ie1 promoter provides about 1000-fold lower levels of transcription than the strong polyhedrin promoter, which provides the high levels of foreign gene transcription typically associated with the use of conventional baculovirus expression vectors (Jarvis et al., 1990a, 1996).

Radioimmunoprecipitation of AcP(+)IESManIII-infected Sf9 cell lysates revealed that the rabbit antiserum prepared for this study precipitated three major proteins with apparent molecular weights of about 132, 125, and 110 kDa (Figure 1A). The specificity of the antiserum was demonstrated by its inability to immunoprecipitate these proteins from mock- or wild-type baculovirus-infected cells and by the fact that none of these proteins were immunoprecipitated with preimmune serum (Figure 1A). These results were confirmed and extended by immunoblotting assays, which showed that the rabbit antiserum specifically reacted with these same three proteins in AcP(+)IESManIII- but not in wild-type baculovirus-infected Sf9 cell lysates (Figure 1B). The strong immunoreactive bands below the 68-kDa marker are nonspecific, as evidenced by their presence in the wild-type baculovirus-infected controls. The results of these experiments established that at least three different forms of SfManIII are produced in Sf9 cells.

Fig. 1. Biosynthesis of SfManIII. (A) Mock-infected, AcP(+)IESManIII-infected (Man III), or wild-type baculovirus-infected (WT) Sf9 cells were pulse labeled with 100 µCi/ml of 35S-Translabel in methionine-free Grace’s medium from 20–24 h postinfection. Intracellular fractions were prepared, immunoprecipitated with preimmune serum (lanes 1) or rabbit anti-SfManIII (lanes 2) as described in Materials and methods, and analyzed by SDS–PAGE and autoradiography. (B) Intracellular fractions were prepared from wild-type baculovirus-infected (WT) or AcP(+)IESManIII-infected (Man III) Sf9 cells at various times postinfection (given in h above each lane) and analyzed by SDS–PAGE and immunoblotting, as described in Materials and methods. The primary antibody was a polyclonal, monospecific rabbit antiserum prepared against SfManIII, as described in Materials and methods. The numbers and arrows to the left of the figure indicate the positions and sizes of molecular weight standards, in kDa. The numbers and arrows to the right of the figure indicate the sizes and positions of the major, specifically immunoreactive forms of SfManIII.
Processing of SfManIII

Pulse-chase experiments were performed to investigate the relationships among these three different forms of SfManIII. The results showed that the 132-kDa protein is the first form of SfManIII to be produced in Sf9 cells (Figure 2A, lane 0). Tiny amounts of the 125- and 110-kDa proteins were first detected after a 2-h chase (Figure 2A, lane 120) and, with increasing chase time, the amounts of these proteins increased at the expense of the 132 kDa protein (Figure 2A, lanes 240 and 480). However, significant amounts of the 132-kDa protein remained even after an 8-h chase (Figure 2A, lane 480). At this time, the 125- and 110-kDa proteins were detectable in the extracellular fraction (Figure 2B). However, only the 110-kDa protein and a new immunoreactive protein of about 105 kDa were detected when the extracellular fraction was analyzed by immunoblotting (Figure 3). These results indicated that SfManIII is initially synthesized as a 132-kDa precursor, which is converted to two intracellular products of 125 and 110 kDa. Both of these products can be secreted, but the 125-kDa protein does not accumulate, whereas the 110-kDa protein does accumulate in the extracellular fraction. The source of the 105-kDa product is unclear. Immunoblotting revealed more of the 105 kDa than the 110 kDa protein in the extracellular fraction (Figure 3), but no 105 kDa protein was detected in the intracellular fraction by either immunoblotting (Figures 1B and 3) or radioimmunoprecipitation (Figures 1A and 2A). These observations suggest that the 110-kDa protein might be slowly converted to the 105-kDa protein in the extracellular fraction. A slow conversion rate is required to explain

Fig. 2. Processing of SfManIII by Sf9 cells. (A) AcP(+)-IESfManIII-infected Sf9 cells were methionine-starved from 14–16 h postinfection and pulse labeled for 5 min with 500 µCi/ml of 35S-Translabel in methionine-free Grace’s medium. The radiolabeling medium was then diluted 1:20 with chase medium, the cells were pelleted and resuspended in chase medium, and equivalent samples were taken immediately (0) and at various times after starting the chase (given in min above each lane). Intracellular fractions were then prepared, immunoprecipitated with rabbit anti-SfManIII, and analyzed by SDS–PAGE and autoradiography. (B) Intracellular (IC) and extracellular (XC) fractions were prepared after a 10-min pulse and 480-min chase, immunoprecipitated with rabbit anti-SfManIII, and analyzed by SDS–PAGE and autoradiography. The numbers and arrows to the left indicate the sizes and positions of molecular weight standards, in kDa. The numbers and arrows to the right indicate the sizes and positions of the major specifically immunoreactive forms of SfManIII.

the absence of the 105-kDa product in the extracellular fraction in the pulse-chase experiment (Figure 2B).

SfManIII has seven potential N-glycosylation sites and in vitro translation experiments had previously suggested that it can be N-glycosylated (Jarvis et al., 1997). This raised the possibility that N-glycosylation and/or N-glycan processing might account for some of the differences in the sizes of the three different forms of SfManIII we identified in Sf9 cells. A combined pulse-chase/endoglycosidase experiment was performed to address this possibility (Figure 4). As before, the results revealed that SfManIII is synthesized as a 132-kDa precursor, which is slowly converted to 125- and 110-kDa products in the cell. The 132-kDa precursor was sensitive to endoglycosidase H, as treatment with this enzyme produced a smaller protein of about 120 kDa. In contrast, both the 125- and the 110-kDa products were resistant to endoglycosidase H. These results confirmed that SfManIII is N-glycosylated in Sf9 cells and revealed that the 132-kDa precursor has only immature, high-mannose N-glycans, whereas both end products have at least some processed N-glycans. The 120-kDa protein produced by treating the 132 kDa precursor with endoglycosidase H comigrated with the SfManIII apoprotein produced in the presence of tunicamycin. This result supported the conclusion that the 132-kDa precursor has no endoglycosidase H–resistant N-glycans. In addition, peptide:N-glycosidase F treatment reduced the sizes of both the 125-kDa and the 110-kDa proteins (data not shown), supporting the conclusion that each is N-glycosylated.

Roles of N-glycosylation and N-glycan processing in production of SfManIII

The finding that SfManIII is N-glycosylated led us to question how N-glycosylation and N-glycan processing might influence the behavior of this protein in Sf9 cells. To address this question, we evaluated the effects of N-glycosylation or N-glycan
 processing inhibitors on the production of SfManIII activity and protein by Sf9 cells.

The results of pNP-α-Man assays showed that low levels of endogenous class II α-mannosidase activity were detectable in the intracellular and extracellular fractions of wild-type baculovirus-infected Sf9 cells, but substantially higher levels were detected in both fractions of AcP(+)IESfManIII-infected cells, as expected (Figure 5). These assays were performed in the presence of cobalt chloride, as preliminary experiments had shown that the activities observed in both fractions of AcP(+)IESfManIII-infected cells were activated by cobalt and inhibited by swainsonine (data not shown). Thus both fractions appeared to contain SfManIII protein(s) with similar if not identical enzymatic activities.

When AcP(+)IESfManIII-infected Sf9 cells were treated with tunicamycin, no SfManIII activity was found in either the intracellular or extracellular fraction (Figure 5). The tunicamycin concentration used in these experiments did not inhibit total protein synthesis by these cells (Jarvis and Summers, 1989) but clearly altered their SfManIII protein profiles, because only a single protein of about 120 kDa was immunoprecipitated from the intracellular fraction, and no protein was specifically immunoprecipitated from the extracellular fraction (Figure 6). The same results were obtained when the intracellular and extracellular fractions were analyzed by immunoblotting (Figure 7). These results showed clearly that N-glycosylation is required for the production and secretion of active SfManIII by AcP(+)IESfManIII-infected Sf9 cells.

When AcP(+)IESfManIII-infected Sf9 cells were treated with castanospermine, significantly more SfManIII activity was found in the intracellular fraction and significantly less was found in the extracellular fraction (Figure 5). This inhibitor also altered the SfManIII protein profile, as the major product immunoprecipitated from both fractions of castanospermine-treated cells was an endoglycosidase H–sensitive protein that migrated slightly behind the 132-kDa SfManIII precursor (Figure 6). In contrast to the enzyme activity levels, the intracellular and extracellular fractions of the untreated and castanospermine-treated cells contained about the same amounts of newly synthesized SfManIII protein (Figure 6). However, immunoblotting analysis showed that the accumulated protein profiles in both fractions were quite different (Figure 7). The intracellular fraction contained not only the protein that migrated slightly behind the 132-kDa protein but also a protein that migrated slightly behind the 110-kDa protein. The extracellular fraction contained no specifically immunoreactive proteins.

Together, these results indicated that N-glycan processing is not required for the production of enzymatically active SfManIII, but it is required for the production of the 125-kDa form of this protein and for the appearance of normal levels of enzymatically active SfManIII protein(s) in the extracellular fraction. The absence of the 110-kDa form of SfManIII in the radioimmunoprecipitations and its presence in the immunoblots from castanospermine-treated cells suggest that this protein is produced more slowly in the absence of N-glycan processing.

Discussion

The purpose of this study was to examine the biosynthesis and processing of SfManIII in Sf9 cells. SfManIII is a Golgi-localized class II α-mannosidase that differs from Golgi α-mannosidase II, the archetype class II processing enzyme, in several significant
to Man3GlcNAc in null mice lacking Golgi emerging subclass of class II presented an excellent opportunity to learn more about this. Thus the availability of this cDNA activity remains unclear and, to the best of our knowledge, no no.

However, the nature of the mouse enzyme(s) that provides this compensated for the absence of Golgi revealed an alternate N-glycan processing pathway in which newly termed α-mannosidase III gene other than the SfManIII cDNA has further processed to complex structures, we have speculated that SfManIII functions in N-glycan processing in Sf9 cells (Kawar et al., 2001). SfManIII is probably an insect ortholog of a cobalt-activated α-mannosidase that converts Man6GlcNAc2 to Man6GlcNAc in null mice lacking Golgi α-mannosidase II (Chui et al., 1997). The creation and analysis of these null mice revealed an alternate N-glycan processing pathway in which this enzymatic activity, newly termed α-mannosidase III, compensated for the absence of Golgi α-mannosidase II. However, the nature of the mouse enzyme(s) that provides this activity remains unclear and, to the best of our knowledge, no α-mannosidase III gene other than the SfManIII cDNA has been identified to date. Thus the availability of this cDNA presented an excellent opportunity to learn more about this emerging subclass of class II α-mannosidases.

The results obtained in this study show that SfManIII is initially synthesized as a high-mannose glycoprotein precursor that is slowly converted to at least two different products. N-glycan trimming is one event that contributes to the appearance of these products, because both have at least some endoglycosidase H-resistant N-glycans, whereas the precursor has none. The size, endoglycosidase H resistance, and intracellular accumulation of the 125-kDa product suggest that it might be the major Golgi-localized form of the enzyme (Kawar et al., 2001). However, small amounts of this protein were also found in the extracellular fraction, indicating that, if it is the Golgi form, it is not completely retained by the Golgi apparatus of ways. SfManIII is activated by cobalt, it can convert Man6GlcNAc2 directly to Man6GlcNAc2, and it cannot hydrolyze GlcNAcMan6GlcNAc2. However, SfManIII also shares some characteristics with Golgi α-mannosidase II, such as its neutral pH optimum, swainsonine sensitivity, and Golgi localization. Based on these properties and the fact that Man6GlcNAc2 can be elongated by N-acetylglucosaminyltransferase I (Altmann et al., 1993; Reck et al., 1995) and further processed to complex structures, we have speculated that SfManIII functions in N-glycan processing in Sf9 cells (Kawar et al., 2001). SfManIII is probably an insect ortholog of a cobalt-activated α-mannosidase that converts Man6GlcNAc to Man6GlcNAc in null mice lacking Golgi α-mannosidase II (Chui et al., 1997). The creation and analysis of these null mice revealed an alternate N-glycan processing pathway in which this enzymatic activity, newly termed α-mannosidase III, compensated for the absence of Golgi α-mannosidase II. However, the nature of the mouse enzyme(s) that provides this activity remains unclear and, to the best of our knowledge, no α-mannosidase III gene other than the SfManIII cDNA has been identified to date. Thus the availability of this cDNA presented an excellent opportunity to learn more about this emerging subclass of class II α-mannosidases.

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enzyme can be proteolytically cleaved to produce a 110-kDa catalytic fragment lacking the transmembrane domain (Moremen and Touster, 1985, 1986; Moremen et al., 1991). On the other hand, SfManIII clearly differs from Golgi α-mannosidase II, because the 110-kDa form of Golgi α-mannosidase II is not produced in vivo and does not accumulate in the extracellular fraction. In addition, the amino acid sequence surrounding the relevant cleavage site in Golgi α-mannosidase II bears no resemblance to any sequence in SfManIII (Moremen et al., 1991; data not shown). Interestingly, with respect to being cleaved, secreted, and accumulating extracellularly, SfManIII behaves more like a glycosyltransferase; many members of this class of enzymes are cleaved in the stem region to yield soluble, extracellular domains with enzymatic activity (reviewed in Varki et al., 1999).

The 110-kDa protein could account for the cobalt-activated, swainsonine-sensitive activity found in the extracellular fraction of AcP(+)-IESfManIII-infected Sf9 cells. Another potential source of this activity is the 105-kDa protein, which might be produced extracellularly from the 110-kDa protein. Though the function of the extracellular forms of SfManIII was not evaluated in this study, we previously demonstrated that a soluble version of this enzyme can cleave terminal α-1,2-linked mannose residues from Manαβ5GlcNAc2 (Kawar et al., 2001). Thus, in addition to the proposed N-glycan processing function of the Golgi-localized form, it is possible that secreted form(s) of SfManIII might also be needed to produce this protein. The major protein detected in castanospermine-treated cells contained significantly less activity than the controls, indicating that N-glycan processing is necessary for efficient secretion of enzymatically active SfManIII. The intracellular activity probably reflects the presence of either or both of two major proteins detected by immunoblotting in castanospermine-treated cells. Based on their electrophoretic mobilities, these proteins appeared to be untrimmed versions of the 132-kDa and 110-kDa forms of SfManIII. The absence of the 125-kDa form of SfManIII in castanospermine-treated cells suggests that N-glycan trimming is the only processing event needed to produce this protein. The major protein detected in castanospermine-treated cells by immunoprecipitation migrated slightly behind the 132-kDa precursor. This method provided no evidence of the other major protein detected by immunoblotting, which migrated slightly behind the 110-kDa product.

Together, the radioimmunoprecipitation and immunoblotting results suggest that the putative proteolytic processing event yielding the smallest form of SfManIII occurs but is retarded when N-glycan trimming is inhibited by castanospermine. Cleavage could be retarded in at least two different ways. The untrimmed precursor might be transported more slowly from the ER to the Golgi apparatus, which is where the putative proteolytic cleavage event would be expected to occur, using the glycosyltransferases as models (reviewed in Paulson and Colley, 1989; Varki et al., 1999). Alternatively, the cleavage site in the untrimmed precursor might be partially occluded by the relatively larger high-mannose N-glycans or a conformational alteration in the protein. Finally, the reduction in extracellular activity reflects the absence of accumulated immunoreactive proteins in the extracellular fraction of castanospermine-treated cells. On the other hand, there was some activity in this fraction, which probably reflects the transient presence of the untrimmed protein migrating slightly behind the 132-kDa form of SfManIII, which was detected by radioimmunoprecipitation but not by immunoblotting analysis of the extracellular fraction. Small amounts of this immature, apparently full-length protein, which appears to be relatively unstable in the extracellular environment, could be secreted by the hypothetical mechanism described previously, independently of the secretory pathway. These mechanisms might also be expected to externalize the untrimmed version of the 110-kDa protein produced in castanospermine-treated cells, but this protein was not observed in the extracellular fraction by either radioimmunoprecipitation or immunoblotting analyses. Perhaps the conformation of the untrimmed version of this protein precludes its externalization.

In summary, this study provided new information on the biosynthesis and processing of an α-mannosidase III. Future
Biosynthesis and processing of SfManIII

Studies will be designed to map the N-glycosylation sites in this protein and determine their individual roles in SfManIII activity. In addition, we hope to elucidate more clearly the processing mechanism by determining the amino terminal sequences of the 110-kDa and 105-kDa forms of SfManIII and identifying the protease responsible for the putative cleavage event.

Materials and methods

Cells and viruses

Sf9 cells were routinely maintained in a shake-flask culture in TNM-FH medium containing 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT) and 1% (w/v) pluronic F68, as described previously (Jarvis et al., 1997). This medium was designated complete TNM-FH. A separate culture was maintained under the same conditions, except these cells were grown in HyQ Sfx-Insect protein free medium (HyClone). Special media used for radiolabeling experiments were supplemented with methionine-free Grace’s medium, which was prepared as described previously (Summers and Smith, 1987), and chase medium, which was methionine-free Grace’s supplemented with 250 µg/ml methionine (Sigma Chemical; St. Louis, MO) and 10 µg/ml cycloheximide (Sigma). For the inhibitor experiments, Grace’s or Sfx-Insect media were supplemented with final concentrations of 5 µg/ml tunicamycin (Calbiochem-Novabiochem; San Diego, CA) or 0.4 mM castanospermine (Calbiochem).

Autographa californica multicapsid nucleopolyhedrovirus strain E2 (Smith and Summers, 1978) was the wild-type baculovirus used in this study, and AcSfManIII was a recombinant baculovirus used to express the full-length SfManIII protein under the control of the polyhedrin promoter, as described previously (Jarvis et al., 1997; the name of this virus was changed from AcSfManII to AcSfManIII after it was found that the Sf9 cDNA encoded an α-mannosidase III). AcP(+)IESfManIII was a recombinant baculovirus used to express the full-length SfManIII protein under the control of the ie1 promoter. To isolate this latter virus, a 3.46-kb BglII–NotI fragment of pSfManII (Jarvis et al., 1996) was subcloned into the BglII–NotI sites downstream of the ie1 promoter in pAcP(+)/ETV3 (Jarvis et al., 1996). The resulting transfer plasmid, designated pAcP(+)IESfManII, was isolated from a 200-ml transformed Escherichia coli culture by alkaline lysis (Birnboim and Doly, 1979) and isopycnic ultracentrifugation on CsCl–ethidium bromide density gradients (Sambrook et al., 1989). The purified transfer plasmid was mixed with Bsu36I-digested BakPak6 baculoviral DNA (Kitts and Possee, 1993) and the mixture was used to cotransfect Sf9 cells by a modified calcium phosphate method (Summers and Smith, 1987). The budded virus produced by the transfected cells was harvested 5 days later and resolved by plaque assay on Sf9 cells. Recombinants were identified by their occlusion-positive plaque phenotypes. Four plaques were picked, purified through two additional rounds of plaque assay, and screened for the ability to produce SfManIII activity in infected Sf9 cells. One clone that had this capability was designated AcP(+)IESfManIII and amplified in Sf9 cells. Working virus stocks were low passage stocks, typically passage 2–4, that had been produced using low multiplicities of infection and titered by plaque assays in Sf9 cells, as described previously (Summers and Smith, 1987).

Production of a polyclonal, monospecific rabbit antiserum against SfManIII

Sf9 cells from cultures maintained in protein-free medium were seeded at a density of 7.5 × 10⁶ cells/flask into six 75-cm² culture flasks (Corning) and infected at a multiplicity of 5 plaque forming units per cell with AcSfManIII. The viral inocula were removed at 1 h postinfection, and the cells were fed with Sfx-Insect and returned to a 28°C incubator. At 48 h postinfection, the media were removed, the cells were extracted for 20 min on ice with cold extraction buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1% Nonidet-P40), and the insoluble fraction was pelleted in a microcentrifuge (Model 235C; Fisher Scientific; Houston, TX) at 4°C. This pellet was an enriched source of SfManIII; preliminary experiments have established that this protein resides in a nonionic detergent-insoluble fraction at late times after infection of Sf9 cells with AcSfManIII (data not shown). The pellet was resuspended in Laemmli disruption buffer (50 mM Tris–HCl, pH 6.8, 4% SDS, and 4% β-mercaptoethanol; Laemmli, 1970), trituated through a 22-gauge needle, boiled for 3 min, and quenched on ice. The entire extract from 45 × 10⁶ infected cells was then loaded onto a 10-cm preparative 5.5% SDS-polyacrylamide gel (Model 491 Prep Cell; Bio-Rad Laboratories; Hercules, CA) and electrophoresed for about 4 h at 12 W constant power. As proteins ran off the bottom of the gel, they were washed into a fraction collector at a flow rate of 0.75 ml/min. Fractions were collected every 2 min, and samples of each were analyzed for SfManIII by SDS–PAGE and Coomassie blue staining.

Peak fractions were pooled and concentrated, and low-molecular-weight contaminants were removed by ultrafiltration, first on Centricon 30, then on Centricon 100 units, according to the manufacturer’s protocols (Amicon; Beverly, MA). The protein concentration in the final preparation was determined by the bicinchoninic acid method (Smith et al., 1985), with bovine serum albumin as the standard, and protein content was examined by SDS–PAGE and Coomassie blue staining. A preparation containing only a single major band of SfManIII was used as the immunogen to produce polyclonal, monospecific rabbit antiserum. This work was performed at Texas A&M University, under a protocol approved by the Institutional Animal Care and Use Committee.

Three New Zealand White rabbits were each given intramuscular injections at two sites with a total of 350 µl of an emulsification containing about 90 µg of the purified protein and an equal volume of RIBI adjuvant (RIBI ImmunoChem Research; Hamilton, MT) in 0.14 M NaCl. The rabbits were boosted 28 days later with the same antigen given by the same method. Two weeks later, the rabbits were bled from the marginal ear vein, and serum samples were tested by immunoblotting for specific antibodies against SfManIII. All three rabbits had anti-SfManIII at titers of at least 1:10,000 and were euthanized and exsanguinated according to the animal protocol.

Analyses of SfManIII biosynthesis and processing

Biosynthesis and processing of SfManIII was analyzed by radioimmunoprecipitation and immunoblotting analyses of
samples from Sf9 cells infected with AcP(+)/IESfManIII, as described previously (Jarvis and Summers, 1989; Jarvis and Garcia, 1994). Generally, Sf9 cells from maintenance cultures in complete TNM-FH or Sfx-Insect were seeded into various culture vessels at low densities and mock-infected, infected with wild-type baculovirus, or infected with AcP(+)/IESfManIII at a multiplicity of infection of 3–5 plaque forming units per cell. The precise pulse labeling, chase, and inhibitor conditions used for individual experiments are detailed in the figure legends. Intracellular fractions were prepared by squirting adherent cells into the growth medium, centrifuging for 1 min in a microcentrifuge at 4°C, treating the cell pellets with cold extraction buffer for 10 min on ice, clarifying the lysate for 5 min at 4°C in a microcentrifuge, and harvesting the supernatant. Extracellular fractions were the cell-free media harvested after low-speed centrifugation of the cells, as described. Intracellular and extracellular fractions were immunoprecipitated with preimmune serum or rabbit anti-SfManIII at a dilution of 1:200, as described previously (Jarvis and Summers, 1989). Immunoblotting assays were performed with preimmune serum or rabbit anti-SfManIII at a dilution of 1:200 as the primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase (Promega; Madison, WI) at a dilution of 1:2000 as the secondary antibody, as described previously (Kawar et al., 2001).

For endoglycosidase treatments, SfManIII was immunoprecipitated and immune complexes were harvested by absorption to S. aureus Cowan I (Kessler, 1975). Immune complexes were washed twice with wash buffer (50 mM Tris–HCl, pH 8.0; 100 mM NaCl, 1% Nonidet-P40, 0.1% SDS, and 1% sodium deoxycholate), once with a commercial endoglycosidase H buffer (NEB G5; New England Biolabs; Boston, MA) diluted 1:10, and disrupted by boiling for 3 min in aqueous 0.5% SDS and 0.1 M β-mercaptoethanol. The S. aureus was pelleted for 5 min in a microcentrifuge at 4°C and the supernatants were split into two equal aliquots. Finally, NEB G5 buffer and equal volumes of either water or endoglycosidase H4 (1000 U; New England Biolabs) were added, the samples were digested at 37°C for 2 h, an equal volume of 2× Laemmli sample buffer was added, and the digests were boiled for 3 min and analyzed by SDS–PAGE.

**SfManIII activity assays**

Intracellular and extracellular fractions were prepared from wild-type baculovirus or AcP(+)/SfManIII-infected Sf9 cells, as described, and samples were assayed for SfManIII activity against pNP-α-Man, as described previously (Jarvis et al., 1997), but modified to include 1 mM CoCl2. The samples used for these assays were normalized according to cell numbers, but, due to a volume restriction, half as much of the extracellular fractions (6.25 × 104 cell equivalents) were assayed, relative to the intracellular fractions (1.25 × 105 cell equivalents).

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