Previously, we cloned and characterized an insect (Sf9) cell cDNA encoding a class II α-mannosidase with amino acid sequence and biochemical similarities to mammalian Golgi α-mannosidase II. Since then, it has been demonstrated that other mammalian class II α-mannosidases can participate in N-glycan processing. Thus, the present study was performed to evaluate the catalytic properties of the Sf9 class II α-mannosidase and to more clearly determine its relationship to mammalian Golgi α-mannosidase II. The results showed that the Sf9 enzyme is cobalt-dependent and can hydrolyze \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) to \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \), but it cannot hydrolyze \( \text{GlcNAcMan}_{\alpha}\text{GlcNAc}_{\kappa} \). These data establish that the Sf9 enzyme is distinct from Golgi α-mannosidase II. This enzyme is not a lysosomal α-mannosidase because it is not active at acidic pH and it is localized in the Golgi apparatus. In fact, its sensitivity to swainsonine distinguishes the Sf9 enzyme from all other known mammalian class II α-mannosidases that can hydrolyze \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \). Based on these properties, we designated this enzyme Sf9 α-mannosidase III and concluded that it probably provides an alternate N-glycan processing pathway in Sf9 cells.

Glycoproteins constitute a large and diverse group of macromolecules with central roles in many biological processes. Among them are the ASn-linked glycoproteins, which consist of a polypeptide backbone and an amide-linked oligosaccharide side chain or N-glycan. The N-glycan precursor, \( \text{Glc}_{\alpha}\text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \), is initially transferred to newly synthesized proteins in the lumen of the endoplasmic reticulum (ER) and is converted to various mature forms by processing enzymes distributed along the secretory pathway. Processing begins with cleavage of the three glucose residues by ER α-glucosidases, which produce \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \), generally known as a “high mannose” N-glycan. In higher eukaryotes, high mannose N-glycans may be further processed by ER and Golgi α-mannosidas, which cleave up to six mannose residues. Cleavage of these mannose residues is required before N-glycans can be converted to “hybrid” or “complex” N-glycans by various glycosyltransferases.

Three types of α-mannosidases are involved in N-glycan processing: endo-α-mannosidase, class I α-mannosidases, and class II α-mannosidases (2–5). Endo-α-mannosidase cleaves the reducing terminus of the mannose residue to which the glucose residues are attached if a glycoprotein containing \( \text{Glc}_{\alpha}\text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) exits the ER. Class I α-mannosidases cleave the four \( \alpha1,2 \)-linked mannose residues, converting \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) to \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) in the ER and Golgi complex. Historically, the only class II α-mannosidase thought to be involved in N-glycan processing was Golgi α-mannosidase II. This enzyme requires the prior action of N-acetylglucosaminyltransferase I and cleaves the terminal \( \alpha1,3 \)- and \( \alpha1,6 \)-linked mannose residues from \( \text{GlcNAcMan}_{\alpha}\text{GlcNAc}_{\kappa} \) producing \( \text{GlcNAcMan}_{\alpha}\text{GlcNAc}_{\kappa} \). Golgi α-mannosidase II was considered to be essential for the conversion of N-glycan precursors to complex structures. However, when the mouse Golgi α-mannosidase II gene was inactivated, most cell types still produced complex N-glycans (6). This observation revealed that mice have at least one additional processing class II α-mannosidase, which compensated for the absence of Golgi α-mannosidase II. Furthermore, although null mouse cell extracts could not hydrolyze \( \text{GlcNAcMan}_{\alpha}\text{GlcNAc}_{\kappa} \), they could convert \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) to \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \). This suggested that N-glycan processing was mediated by an α-mannosidase activity, termed α-mannosidase III, which produced a novel \( \text{Man}_{\alpha}\text{GlcNAc}_{\kappa} \) intermediate in these cells. However, the enzyme(s) responsible for this activity were not purified, nor was the gene encoding this enzyme identified.

In a previous study, we cloned and characterized a cDNA encoding a class II α-mannosidase from the lepidopteran insect cell line, Sf9 (7). The deduced amino acid sequence of this enzyme is similar to those of various mammalian Golgi α-mannosidases II. Like the latter enzymes, the Sf9 α-mannosidase is an integral membrane glycoprotein with type II topology. It can hydrolyze \( p \)-nitrophenyl \( \alpha-\text{D}-\text{mannopyranoside} \) (pNP-\( \alpha \)-Man), and it is inhibited by swainsonine. In the present study, we further examined the catalytic properties of this Sf9 class II α-mannosidase to more clearly determine its relationship to mammalian Golgi α-mannosidase II. The results showed that this enzyme is actually distinct from Golgi α-mannosidase II as its activity is stimulated by cobalt and it can hydrolyze various substrates containing terminal mannose residues but not \( \text{GlcNAcMan}_{\alpha}\text{GlcNAc}_{\kappa} \). In view of these properties, we have designated this enzyme Sf9 α-mannosidase III (SfManIII), and we propose that it functions in an alternate N-glycan processing pathway in Sf9 cells.
Isolation of a Recombinant Baculovirus Expressing a GFP-tagged Form of SfManIII—A cDNA fragment encoding full-length SfManIII was subcloned into the immediate early baculovirus transfer plasmid, pAcP+ EITV3 (18). The sequence encoding enhanced green fluorescent protein, a mutant form of GFP with enhanced fluorescence at 507 nm (CLONTECH, Palo Alto, CA), was then inserted immediately downstream, in-frame with the SfManIII coding sequence. The resulting plasmid, pAcP+ E1-I-SfManIII-GFP, encoded a hybrid protein consisting of the full-length SfManIII protein with GFP fused to its C terminus under the transcriptional control of a baculovirus immediate early promoter. This plasmid was used to produce a recombinant baculovirus, AcP+ E1-I-SfManIII-GFP, which expresses the enhanced green fluorescent protein-tagged SfManIII protein during the immediate early phase of infection. AcP+ E1-I-SfManIII-GFP was produced by homologous recombination (9, 11) with Bsu36I-digested BacPAK6 viral DNA (12), plaque-purified twice, amplified in Sf9 cells, and titrated by plaque assay (9).

Confocal Microscopy—Sf9 cells were seeded at a density of 300,000 cells/chamber into LAB-TEK® two-chamber slides (Nalge Nunc International Corp., Naperville, IL), infected with 3 plaque-forming units/cell of AcP+ E1-I-SfManIII-GFP, and cultured for 18 h in TNM-FH medium containing 10% (v/v) heat-inactivated fetal bovine serum. The cells were then incubated with either Golgi- or lysosome-specific dyes, rinsed with phosphate-buffered saline, and examined with a Leica TSD-4D CSLM confocal microscope (Leica, Heidelberg, Germany). To stain the Golgi, the cells were treated for 40–120 min at 27 °C with Grace’s medium containing 0.5% (v/v) heat-inactivated fetal bovine serum and 250 nM BODIPY™-TR ceramide (Molecular Probes Inc., Eugene, OR). To stain the lysosomes, the cells were treated for 4 h in room temperature with Grace’s medium containing 0.5% (v/v) heat-inactivated fetal bovine serum and 25 nM LysoTracker™ Red DND-99 (Molecular Probes).

RESULTS

Expression and Purification of GST-SfManIII—AcGST-SfManIII is a recombinant baculovirus that encodes a GST-tagged, secreted form of SfManIII. Sf9 cells were infected with either this recombinant virus or a wild type control, and the extracellular media were harvested and analyzed by immunoblotting with rabbit anti-GST as described under “Experimental Procedures.” The results revealed that there were two specifically immunoreactive proteins in the extracellular growth medium from the AcGST-SfManIII-infected cells (Fig. 1A, lane 1). Neither was detected in the medium from wild type virus-infected cells (data not shown). The major protein had an apparent molecular mass of ~150 kDa suggesting that it is intact GST-SfManIII, which has a calculated molecular mass of 157.5 kDa. The other, relatively minor protein had an apparent molecular mass of ~130 kDa and is probably a degradation product. Another, weakly immunoreactive protein of ~68 kDa was also detected, but its presence in the negative controls (data not shown) indicated that it was not specific. Coomassie Blue staining revealed that the growth medium contained at least three proteins, including the 68-kDa protein, which were present in vast excess relative to the 150- and 130-kDa immunoreactive proteins (Fig. 1B, lane 1).

Affinity chromatography on an immobilized glutathione column was used to isolate the extracellular immunoreactive proteins as described under “Experimental Procedures.” The affinity column retained the specifically immunoreactive proteins detected in the growth medium (compare Fig. 1A, lanes 1 and 4), but not the nonspecifically immunoreactive and non-immunoreactive proteins as determined by Coomassie Blue staining (compare Fig. 1B, lanes 1 and 4). About half of the α-mannosidase activity in the medium was retained by the column as determined by pNP-α-Man assays (data not shown). Multiple attempts to elute active GST-SfManIII from the column with reduced glutathione were unsuccessful, but the column-bound enzyme had high levels of pNP-α-Man activity (data not shown). Therefore, the immobilized glutathione-agarose beads were removed from the column, resuspended in buffer A, and used as the source of GST-SfManIII for this study. The bound
GST-SfManIII retained its activity for at least 1 week at 4 °C but was inactivated by freezing.

The Coomassie Blue-stained gel profile of an excess amount of the material extracted from the immobilized glutathione-agarose beads is shown in lane 5 of Fig. 1B. This analysis revealed that the SfManIII preparations consisted of a major protein with an apparent molecular mass of ~150 kDa, a minor protein with an apparent molecular mass of ~130 kDa, and no other detectable proteins. Presumably, the two proteins stained by Coomassie Blue corresponded to those detected by immunoblotting.

Biochemical Characterization of GST-SfManIII—A series of enzyme assays was performed to characterize the biochemical properties of GST-SfManIII. One set of assays was designed to evaluate the influence of various metal ions on GST-SfManIII activity (Table I). The addition of 1 mM EDTA reduced hydrolysis of pNP-α-Man to less than 10% of control levels indicating that GST-SfManIII has a divalent cation requirement. Indeed, GST-SfManIII activity was greatly enhanced by the addition of CoCl₂, MnCl₂, NiCl₂, and, to a lesser extent, MnCl₂. NiCl₂ had little effect, and GST-SfManIII activity was inhibited by ZnCl₂. The effect of calcium was not examined because TNM-FH and Grace’s medium contain high concentrations of CaCl₂.

Another set of assays was performed to examine the influence of pH on GST-SfManIII activity against pNP-α-Man in the presence of 1 mM CoCl₂ (Fig. 2). GST-SfManIII activity was negligible at pH 5.5, increased at pH 5.7 and 5.9, decreased at pH 6.1, and finally rose to a plateau at pH 6.5–6.7. These data showed that GST-SfManIII had significantly more activity around neutral pH than at acidic pH, which is consistent with the idea that it is a processing, not a lysosomal, enzyme. The reason for the reproducible decline in activity observed at pH 6.1 is unknown.

Finally, a set of pNP-α-Man assays was performed to determine the effect of swainsonine on GST-SfManIII activity (Fig. 3). GST-SfManIII was clearly sensitive to swainsonine in assays performed at pH 6.3 in the presence of 1 mM CoCl₂. The IC₅₀ was ~10 nM, and 1.5 μM swainsonine reduced GST-SfManIII activity to less than 0.5% of control values.

Thus, GST-SfManIII shares some biochemical properties with Golgi α-mannosidase II including its neutral pH optimum and swainsonine sensitivity, but its dependence on cobalt clearly distinguishes this enzyme from Golgi α-mannosidase II.

Substrate Specificity of GST-SfManIII—Whereas it was well established that Golgi α-mannosidase II hydrolyzes GlcNAcMan₉GlcNAc₂ to GlcNAcMan₈GlcNAc₂ (2), the natural substrate specificity of SfManIII had not been determined. Therefore, experiments were undertaken to examine the action of GST-SfManIII on various glycan substrates. GST-SfManIII had little effect on GlcNAcMan₉GlcNAc₂-PA even after extended incubation times (Fig. 4). Thus, GST-SfManIII failed to hydrolyze the natural substrate of Golgi α-mannosidase II.

On the other hand, GST-SfManIII effectively hydrolyzed Man₉GlcNAc₂-PA through a Man₇GlcNAc₂-PA intermediate to Man₅GlcNAc₂-PA (Fig. 5). This activity was not due to contamination with endogenous α-mannosidases as there was no detectable hydrolysis when Man₅GlcNAc₂-PA was incubated for 72 h with a mock affinity-purified enzyme preparation from wild type baculovirus-infected Sf9 cells (Fig. 5, Mock). Thus, GST-SfManIII can hydrolyze the proposed substrate for α-mannosidase III, the enzyme thought to mediate the alternate N-glycan processing pathway in mice (6).

Considering its unusual substrate specificity, we also evaluated the ability of GST-SfManIII to hydrolyze other glycans, which had terminal α,1,2-linked mannose residues (Fig. 6). GST-SfManIII was incubated for 72 h with Man₈GlcNAc₂-PA, Man₇GlcNAc₂-PA (mixed isomers) or Man₆GlcNAc₂-PA (isomer C with one α,1,2-linked mannose residue on the middle arm). Over half of the Man₆GlcNAc₂-PA was consumed producing Man₅GlcNAc₂-PA and a small amount of Man₄GlcNAc₂-PA. Similarly, most of the Man₅GlcNAc₂-PA was consumed producing Man₄GlcNAc₂-PA and small amounts of Man₃GlcNAc₂-PA and Man₂GlcNAc₂-PA. Finally, Man₄GlcNAc₂-PA was completely consumed producing mainly Man₃GlcNAc₂-PA and small amounts of Man₂GlcNAc₂-PA and Man₁GlcNAc₂-PA. None of these activities were due to contaminating endogenous α-mannosidases as there was no detectable hydrolysis of any of these substrates when they were incubated for 72 h with a mock affinity-purified enzyme preparation from wild type baculovirus-infected Sf9 cells (Fig. 6, Mock column).

These results demonstrated that GST-SfManIII can hydrolyze a variety of glycan substrates containing terminal α,1,2-linked mannose residues in addition to Man₈GlcNAc₂-PA. Interestingly, SfManIII produced much less Man₇GlcNAc₂-PA from the Man₆GlcNAc₂-PA substrate than from the Man₅GlcNAc₂-PA (mixed isomer) substrate. The reason why Man₇GlcNAc₂-PA was not completely converted to Man₇GlcNAc₂-PA is unclear.
is possible that this simply reflects the relatively slow conversion of Man$_9$GlcNAc$_2$-PA to Man$_8$GlcNAc$_2$-PA (Fig. 6, top left panel). Alternatively, it is possible that this reflects the composition of the Man$_8$GlcNAc$_2$-PA (mixed isomer) substrate, which consisted of 75% isomer A, 20% isomer B, and 5% isomer C (data not shown). This mixture might be a better substrate for SfManIII because isomers A and B are better substrates for SfManIII. In contrast, SfManIII digestion of Man$_9$GlcNAc$_2$-PA could yield a totally different mixture of Man$_8$GlcNAc$_2$-PA isomers, which is relatively resistant to further digestion by SfManIII. These possibilities were not examined in this study.

All of the assays of GST-SfManIII activity against glycan substrates were performed in the presence of 1 mM CoCl$_2$. Therefore, additional assays were performed to specifically evaluate the cobalt requirement for Man$_9$GlcNAc$_2$-PA hydrolysis by GST-SfManIII. Hydrolysis was greatly reduced when CoCl$_2$ was not added to the assays and was completely abolished in the presence of 5 mM EDTA (data not shown) indicating that cobalt is required for the hydrolysis of Man$_9$GlcNAc$_2$-PA by GST-SfManIII. The effect of swainsonine on Man$_9$GlcNAc$_2$-PA hydrolysis by GST-SfManIII was also evaluated, and the results were similar to those obtained in the pNP-$\alpha$-Man assays (data not shown) indicating that hydrolysis of Man$_9$GlcNAc$_2$-PA by GST-SfManIII is sensitive to swainsonine, as well.

**Intracellular Distribution of SfManIII-GFP**—All of the properties of SfManIII were consistent with the interpretation that this enzyme plays a role in N-glycan processing in Sf9 cells. If SfManIII has this role in vivo, it should reside in the Golgi compartment. To examine its intracellular distribution, confocal microscopy was used to examine live Sf9 cells infected with a baculovirus vector encoding a full-length, GFP-tagged version of SfManIII. This baculovirus expression vector expresses SfManIII-GFP under the transcriptional control of a baculovirus promoter that is active immediately after infection and provides relatively low expression levels (18). This promoter was used to circumvent the potential problem of aberrant localization of SfManIII-GFP, which might happen if it had been overexpressed. Sf9 cells were infected with this baculovirus for 18 h, and then labeled with the red fluorescent dyes BODIPY®TR ceramide to stain the Golgi (Fig. 7, top panels) or LysoTracker™ Red DND-99 to stain the lysosomes (Fig. 7, bottom panels). Confocal microscopy revealed that the SfManIII-GFP was localized in punctate fluorescent structures dispersed throughout the cytoplasm but excluded from the nuclei.
This staining pattern overlapped significantly with the pattern obtained with the Golgi dye (Fig. 7, top middle panel) but not with the pattern obtained with the lysosomal dye (Fig. 7, bottom middle panel). It is noteworthy that a previous study established the dispersed nature of the Golgi elements in Sf9 cells (19). Thus, the confocal microscopy results described above indicate that SfManIII is localized in the Golgi compartment of Sf9 cells.

**DISCUSSION**

This study demonstrated that Sf9 cells encode a class II \( \alpha \)-mannosidase that is distinct from Golgi \( \alpha \)-mannosidase II and has a unique set of properties. Based on these properties, and in accordance with the nomenclature used by Chui et al. (6), we named this enzyme SfManIII. Unlike Golgi \( \alpha \)-mannosidase II, SfManIII is activated by cobalt, it can hydrolyze Man\(_{5}\)-GlcNAc\(_2\), and it can convert Man\(_{5}\)-GlcNAc\(_2\) to Man\(_{3}\)-GlcNAc\(_2\) but cannot hydrolyze GlcNAcMan\(_{5}\)-GlcNAc\(_2\). On the other hand, SfManIII has the same neutral pH optimum and intracellular distribution as Golgi \( \alpha \)-mannosidase II. These properties, together with the fact that Man\(_{5}\)-GlcNAc\(_2\) can be elongated by N-acetylglucosaminyltransferase I (20, 21) and further processed to complex structures, suggest that SfManIII functions in N-glycan processing in Sf9 cells. However, the current study did not establish whether or not SfManIII actually plays this role in vivo.

The early steps of N-glycan processing, including mannose trimming, appear to be similar or identical in Sf9 and mammalian cells (22, 23). A cDNA encoding a class I \( \alpha \)-mannosidase has been cloned from Sf9 cells, and its product has been extensively characterized (19, 24, 25). The results of these studies have shown that SfManI is virtually identical in structure, function, and intracellular distribution to the mammalian \( \alpha \)-mannosidases IA and IB. In addition, Sf9 cells have a class II \( \alpha \)-mannosidase that acts on GlcNAcMan\(_{5}\)-GlcNAc\(_2\) without a metal ion requirement and appears to be functionally identical to mammalian Golgi \( \alpha \)-mannosidase II (26). In contrast, whereas SfManIII has the biochemical properties of Class II mannosidases, a mammalian ortholog with similar catalytic characteristics has not yet been purified or cloned (5).

Considering the similarities between the other processing \( \alpha \)-mannosidases of Sf9 and mammalian cells, there is probably a mammalian ortholog of SfManIII as well. One likely candidate is the cobalt-activated \( \alpha \)-mannosidase activity(ies) that convert Man\(_{5}\)-GlcNAc to Man\(_{3}\)-GlcNAc in null mice lacking Golgi \( \alpha \)-mannosidase II (6). This activity, termed \( \alpha \)-mannosidase III, compensates for the absence of Golgi \( \alpha \)-mannosidase II in these mice by providing an alternate pathway for N-glycan processing. However, it is currently impossible to unequivocally decipher the relationship between this activity and SfManIII because the mouse enzyme(s) that provide this activity have not yet been identified. In fact, several enzymes probably contribute to the \( \alpha \)-mannosidase III activity detected in mouse cell lysates including both swainsonine-resistant enzymes that are not involved in N-glycan processing, as well as one or more...
swainsonine-sensitive, processing enzymes.

One mammalian enzyme that could account for the Man$_5$GlcNAc$_2$-hydrolyzing activity in the null mouse cell lysates is an α-mannosidase first characterized in BHK cells (27) and later purified from rat liver (28, 29). This BHK/rat liver enzyme is cobalt-activated, relatively resistant to swainsonine, and can convert Man$_5$GlcNAc$_2$ to Man$_5$GlcNAc$_1$ but cannot hydrolyze pNP-α-Man. This enzyme does not appear to be a processing enzyme because there is no complex N-glycan biosynthesis in BHK cells treated with swainsonine (30) indicating that all of the processing class II α-mannosidases in these cells are swainsonine sensitive. But, like the null mice discussed above, mutant BHK cells lacking detectable Golgi α-mannosidase II activity can still synthesize some complex N-glycans (31). Thus, these cells must have another, swainsonine sensitive α-mannosidase III activity that provides the alternate N-glycan processing pathway.

A second mammalian enzyme that could account for the α-mannosidase III activity is an isozyme of Golgi α-mannosidase II that has been termed α-mannosidase II* (32). This enzyme shares sequence similarity with both the human and insect class II α-mannosidases, but its N-glycan substrate specificity has not yet been characterized.

There also is another group of mammalian enzymes, generally classified as ER/cytosolic α-mannosidases, which could account for the Man$_5$GlcNAc$_2$-hydrolyzing activity detected in the null mouse cell lysates. These enzymes are activated by cobalt, are relatively resistant to swainsonine, and can convert Man$_5$GlcNAc$_2$ to Man$_5$GlcNAc$_1$. However, they act only on glycans with a single GlcNAc residue on their reducing end and appear to function in N-glycan catabolism rather than processing (33–36).

SfManIII is clearly distinct from both the swainsonine resistant BHK/rat liver enzyme and the ER/cytosolic α-mannosidases. SfManIII is sensitive to swainsonine, can hydrolyze pNP-α-Man, and can efficiently hydrolyze glycans with chitobiose cores. Considering these properties and the fact that Sf9 cells have another α-mannosidase, which is functionally equivalent to mammalian Golgi α-mannosidase II (26), SfManIII is probably equivalent to the enzyme providing the alternate pathway in the null mice (6) and mutant BHK cells (31). However, an unequivocal test of this hypothesis awaits purification of the mammalian enzyme(s) responsible for the α-mannosidase III activity.

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Insect Cells Encode a Class II \(\alpha\)-Mannosidase with Unique Properties
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