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Biosynthesis and subcellular localization of a lepidopteran insect α 1,2-mannosidase

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Abstract

Like lower and higher eucaryotes, insects have α 1,2-mannosidases which function in the processing of N-glycans. We previously cloned and characterized an insect α 1,2-mannosidase cDNA and demonstrated that it encodes a member of a family of N-glycan processing α 1,2-mannosidases (Kawar, Z., Herscovics, A., Jarvis, D.L., 1997. Isolation and characterisation of an α 1,2-mannosidase cDNA from the lepidopteran insect cell line Sf9. *Glycobiology* 7, 433–443). These enzymes have similar protein sequences, require calcium for their activities, and are sensitive to 1-deoxymannojirimycin, but can have different substrate specificities and intracellular distributions. We recently determined the substrate specificity of the insect α 1,2-mannosidase, SfManI (Kawar, Z., Romero, P., Herscovics, A., Jarvis, D.L., 2000. N-glycan processing by a lepidopteran insect α 1,2-mannosidase. *Glycobiology* 10, 347–355). Now, we have examined the biosynthesis and subcellular localization of SfManI. We found that SfManI is partially N-glycosylated and that N-glycosylation is dramatically enhanced if the wild type sequon is changed to one that is highly utilized in a mammalian system. We also found that an SfManI-GFP fusion protein had a punctate cytoplasmic distribution in insect cells. Colocalization studies indicated that this fusion protein is localized in the Golgi apparatus, not in the endoplasmic reticulum or lysosomes. Finally, N-glycosylation had no influence over the substrate specificity or subcellular localization of SfManI.

Keywords

α 1,2-Mannosidase; Subcellular; Localization; Insect; GFP; N-glycosylation

1. Introduction

Protein N-glycosylation begins with the transfer of a precursor, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, to proteins as they enter the endoplasmic reticulum (ER), and this N-glycan is converted into its final form by processing enzymes localized along the secretory pathway. Processing is initiated by two ER α -glucosidases, which remove the glucose residues. This is followed by the action of various α 1,2-mannosidases, which can remove one or more of the four α 1,2-linked mannose residues. *Saccharomyces cerevisiae* has only one α 1,2-mannosidase which cleaves a specific mannose residue to produce $\text{Man}_8\text{GlcNAc}_2$. This intermediate is then elongated by the addition of several mannose residues to produce elaborate polymannose N-glycans (reviewed in Herscovics, 1999a). Mammalian cells, on the other hand, have several α 1,2-mannosidases which can cleave all four α 1,2-linked mannose residues to produce $\text{Man}_5\text{GlcNAc}_2$. This intermediate is then processed further to produce complex N-glycans containing N-acetylglucosamine, fucose, galactose, and sialic acid residues (reviewed in

Kornfeld and Kornfeld, 1985). Insect cells are thought to be similar to mammalian cells in this regard, as they clearly have α 1,2-mannosidases which can trim all the α 1,2-linked mannose residues. Insect cells also perform some elongation reactions, but generally cannot produce complex sialylated N-glycans (reviewed in Marz et al., 1995; Jarvis et al., 1998a).

We previously isolated an α 1,2-mannosidase cDNA from the lepidopteran insect cell line, Sf9, and characterized the enzyme it encodes, SfManI (Kawar et al., 1997). SfManI was found to be a member of a family of α 1,2-mannosidases which have similar amino acid sequences, require calcium, and are sensitive to 1-deoxymannojirimycin (reviewed in Moremen et al., 1994 Herscovics, 1999a,b). However, the individual enzymes in this family have different subcellular distributions and substrate specificities. To establish the relationship between SfManI and other known α 1,2-mannosidases, we recently determined the substrate specificity of SfManI (Kawar et al., 2000). Like the mammalian α 1,2-mannosidases IA and IB, SfManI cleaved all four α 1,2-linked mannose residues from Man₉GlcNAc₂ and cleaved the mannose on the middle arm of the oligosaccharide at a slower rate than the other three. However, the sequence of mannose cleavage by SfManI differed from that of the mammalian α 1,2-mannosidases IA and IB.

The purpose of the present study was to characterize the biosynthesis and subcellular localization of SfManI. The deduced amino acid sequence of SfManI includes a single potential N-glycosylation site. Therefore, the first part of this study was designed to determine if SfManI is a glycoprotein, as most α 1,2-mannosidases contain potential N-glycosylation sites (Camirand et al., 1991; Bause et al., 1993; Herscovics et al., 1994; Lal et al., 1994; Kerscher et al., 1995; Tremblay et al., 1998), and many have been shown to be glycoproteins (Tulsiani and Touster, 1988 Camirand et al., 1991; Bieberich and Bause, 1995). In the second part of this study, we investigated the intracellular distribution of SfManI. Some α 1,2-mannosidases are localized in the ER (Roth et al., 1990; Burke et al., 1996; Bieberich et al., 1997), while others are localized in the Golgi apparatus (Velasco et al., 1993; Herscovics et al., 1994; Lal et al., 1994; Bieberich and Bause, 1995). Thus, it was of interest to determine whether SfManI, the best characterized insect α 1,2-mannosidase, is found in the ER or Golgi apparatus of Sf9 cells.

2. Materials and methods

2.1. Cells and cell culture

Sf9 cells are derived from the IPLB-Sf21-AE cell line, which was originally isolated from *Spodoptera frugiperda* (fall armyworm) ovaries (Vaughn et al., 1977). Sf9 cells were maintained as a suspension culture at densities between 0.3 and 3.0 $\times$ 10⁶ cells per ml in TNM-FH medium (Summers and Smith, 1987) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 0.1% (w/v) pluronic F68 (BASF Wym-andotte Corp., Parsippany, NJ; Murhammer and Goochee, 1988).

2.2. Plasmid constructions

- i. PET-SfManI. A fragment of the SfManI cDNA (Kawar et al., 1997) encoding the C-terminal part of the protein was subcloned into pET-17b, an *E. coli* expression plasmid (Novagen Inc., Madison, WI). The resulting construct, which was designated pET-SfManI, encoded amino acids 259–670 of SfManI downstream of a T7 promoter, a bacterial ribosomal binding site, and an in-frame translational initiation codon.
- ii. pAcSfManI. The complete SfManI open reading frame was subcloned into pVL1393 (Webb and Summers, 1990), a baculovirus transfer plasmid. The

resulting construct encoded full-length SfManI under the transcriptional control of the polyhedrin promoter, as previously described (Kawar et al., 1997).

- iii. pAcP $\oplus$ IE1-SfManI-GFP. A fragment of the SfManI cDNA, encoding all but the last seven amino acids of SfManI, was subcloned into pEGFP-N1 (Clontech, Palo Alto, CA), which encodes a mutant form of GFP with enhanced fluorescence at 507 nm. A fragment encoding the SfManI-GFP fusion protein was then excised and subcloned into pAcP $\oplus$ IE1TV3, an immediate early baculovirus transfer plasmid (Jarvis et al., 1996). The resulting construct encoded the SfManI-GFP fusion protein under the transcriptional control of the iel promoter, which provides relatively low-level expression beginning immediately after infection.
- iv. pAcP $\oplus$ IE1-GFP. The complete GFP open reading frame was subcloned into pAcP $\oplus$ IE1TV3 for expression of unfused GFP under the transcriptional control of the iel promoter.
- v. pAcP $\oplus$ IE1-GalT-GFP. The complete bovine β 1,4- galactosyltransferase open reading frame (Shaper et al., 1986) was inserted upstream and in-frame with the GFP open reading frame in pAcP $\oplus$ IE1-GFP. The resulting immediate early baculovirus transfer plasmid encoded a hybrid protein consisting of the full-length β 1,4-galactosyltransferase protein with GFP fused to its C-terminus.

2.3. Recombinant baculovirus production

Recombinant baculoviruses were produced using the transfer plasmids described above by a standard allelic transplacement method (Summers and Smith, 1987; O'Reilly et al., 1992) with *Bsu36I*-digested BacPAK6 viral DNA (Kitts and Possee, 1993) as the target for homologous recombination. The recombinant viruses were plaque purified twice, amplified in Sf9 cells, and titered by plaque assay.

2.4. Site-directed mutagenesis

The DNA sequence encoding the sequon in SfManI was altered by site-directed mutagenesis of pAcSfManI, as described previously (Deng and Nickoloff, 1992). In one mutant, the sequon was eliminated by changing the asparagine residue in the first position to serine. In another mutant, the sequon was changed from Asn-Asp-Ser to Asn-Ser-Thr in an attempt to enhance N-glycosylation at this site. Site-directed mutagenesis was carried out using the TransformerTM Site-Directed Mutagenesis Kit (Clontech) according to the supplier's instructions.

2.5. Rabbit antiserum against SfManI

A fragment of the SfManI protein was produced in *E. coli* transformed with the pET-SfManI plasmid according to standard methods (Studier et al., 1990). Soluble proteins were extracted and the SfManI fragment was purified by size fractionation on a preparative SDS-PAGE apparatus (Prep Cell; Bio-Rad Laboratories, Hercules, CA). Fractions containing the SfManI fragment were combined, concentrated using Centricon® 30 concentrators (Amicon, Beverly, MA), mixed with RIBI adjuvant (RIBI ImmunoChem Research Inc., Hamilton, MT), and injected into rabbits to produce polyclonal monospecific antisera. The specificity of these antisera was confirmed by immunoblotting and radioimmunoprecipitation of extracts from Sf9 cells infected with a baculovirus expression vector encoding SfManI. The major protein detected in both assays by the anti-SfManI antisera was about 68 kDa, which is the expected size of SfManI. This protein was not detected with normal rabbit serum or in Sf9 cells infected with wild type baculovirus. The antiserum from one rabbit was used in all the experiments described in this paper.

2.6. Radioimmunoprecipitations

Sf9 cells were infected with baculovirus expression vectors encoding wild type SfManI or the SfManI N-glycosylation site mutants. The cells were labeled with tran-³⁵S-label from 24 to 28 h post infection, then total intracellular protein extracts were prepared. Some of the infected-cell cultures were treated with 1.5 mg of tunicamycin per ml of medium from 20 to 28 h post infection. SfManI was immunoprecipitated from the total intracellular protein extracts using the anti-SfManI antiserum, as described previously (Jarvis et al., 1998b). One half of each immunoprecipitate was treated with EndoH_f (New England Biolabs Inc., Beverly, MA) and the other half was treated with buffer alone, according to the supplier's instructions. The samples were then analyzed by SDS-PAGE and autoradiography.

2.7. Immunoblotting

Sf9 cells were infected with wild type baculovirus or baculovirus expression vectors encoding unfused GFP or SfManI-GFP. Total intracellular protein extracts were prepared at 24 h post infection and resolved by SDS-PAGE. The proteins were electrophoretically transferred from the polyacrylamide gel to a Westran® membrane (Schleicher & Schuell, Keene, NH Towbin et al., 1979) and the membrane was probed with an anti-GFP antiserum (Clonotech). The primary antibody reaction was followed by a secondary reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Inc., Madison, WI), which was detected using a standard color reaction (Blake et al., 1984).

2.8. Confocal microscopy

Sf9 cells were seeded into LAB-TEK® chamber microscope slides (Nalge Nunc International Corp., Naperville, IL) and infected with wild type baculovirus or baculovirus expression vectors encoding the various GFP constructs described above. At 18 h post infection, the cells were rinsed with TBS (140 mM NaCl; 25 mM Tris-HCl, pH 7.4) and examined using a Leica TSD-4D CSLM confocal microscope (Leica, Heidelberg, Germany). To stain the Golgi, the cells were incubated in medium containing 250 nM BODIPY®TR ceramide (Molecular Probes Inc., Eugene, OR) for 40 min before examination. To stain the ER, the cells were incubated in medium containing 3 nM DiIC6(3) (Molecular Probes) for 20 min before examination, and to stain the lysosomes, they were incubated in medium containing 25 nM LysoTracker™ Red DND-99 (Molecular Probes) for 2 min before examination.

3. Results

3.1. SfManI is partially N-glycosylated

Computer analysis of the deduced amino acid sequence of SfManI revealed that it includes a potential N-glycosylation site in its putative stem region. To determine if this site was actually utilized, a recombinant baculovirus vector was used to express SfManI in Sf9 cells in the presence or absence of tunicamycin, which blocks N-glycosylation. Newly synthesized proteins were radiolabeled with tran-³⁵S-label and SfManI was extracted and immunoprecipitated with a polyclonal antiserum raised against *E. coli*-produced SfManI, as described in Materials and Methods. The immunoprecipitate was then treated with buffer alone or endoglycosidase H_f, which removes high mannose N-glycans from proteins, and analyzed by SDS-PAGE and autoradiography (Fig. 1). A major band of about 68 kDa, which is the expected size of SfManI, was present in all samples. This band was absent in control immunoprecipitations using normal rabbit serum (data not shown), supporting its identification as SfManI. An additional, relatively minor, band was observed just above the major SfManI band in the sample from cells grown in the absence of tunicamycin (Fig. 1, lane 1). This band was abolished by endoglycosidase H_f treatment (Fig. 1, lane 2), and was absent in the samples from cells treated with tunicamycin (Fig. 1, lanes 3 and 4). These

results suggested that the minor band represented an N-glycosylated subpopulation of SfManI. Furthermore, the ability of endoglycosidase H_f to remove this N-glycan indicated that it has a high mannose structure and has not been processed through the mannosidase II step of the N-glycan processing pathway.

3.2. Sequon usage by Sf9 cells conforms with mammalian preferences

Site-directed mutagenesis was used to further investigate N-glycosylation of SfManI. First, we knocked out the N-glycosylation site by changing the asparagine residue in the first position of the sequon to serine. Second, we attempted to enhance N-glycosylation at this site by changing the sequon from Asn–Asp–Ser to Asn–Ser–Thr. A previous study had shown that this latter sequence is the most efficiently used sequon during *in vitro* translation by rabbit reticulocyte lysates supplemented with canine microsomal membranes (Kasturi et al., 1997). The two mutant forms of SfManI were expressed in Sf9 cells, radiolabeled, extracted, and immunoprecipitated as described above. As expected, the minor, slightly larger form of SfManI was absent in cells expressing the knockout mutant (Fig. 2), confirming that this slightly larger protein was an N-glycosylated form of the enzyme. Furthermore, the slightly larger form represented the majority of the SfManI produced by cells expressing the mutant protein with the enhanced sequon. This result indicated that the enhanced sequon was, in fact, more efficiently utilized than wild type.

3.3. Expression of SfManI-GFP hybrid proteins

Our next goal was to determine the intracellular distribution of SfManI. To accomplish this, we constructed a baculovirus expression vector which encodes a hybrid protein consisting of GFP fused to the C-terminus of SfManI (SfManI-GFP), under the transcriptional control of the immediate early 1 (ie 1) promoter. This promoter is active immediately upon infection and provides a relatively low level of expression, which avoids the potential problem of mislocalization due to high-level over-expression. It also is important to note that previous studies have shown that adding GFP to the C-terminus of type II membrane proteins does not alter their localization within the secretory pathway (Cole et al., 1996; Sciaky et al., 1997). GFP fusion constructs were also prepared with the knockout and enhanced N-glycosylation mutants of SfManI. An immediate early baculovirus expression vector encoding unfused GFP, which is a soluble protein that does not enter the secretory pathway, was constructed as a control.

To assess the expression of SfManI-GFP and unfused GFP, Sf9 cells were infected with the baculovirus vectors encoding these proteins or wild type baculovirus. Total protein extracts were prepared from each culture at 24 h post infection and analyzed by SDS–PAGE and immunoblotting with an anti-GFP antiserum (Fig. 3). An immunoreactive band of about 27 kDa, which is the expected size of GFP, was observed in the extracts from cells infected with the baculovirus vector encoding unfused GFP (Fig. 3, lane 2). An immunoreactive band of about 97 kDa, which is the expected size of SfManI-GFP, was observed in extracts from cells infected with the baculovirus expression vector encoding SfManI-GFP (Fig. 3, lane 3). No major immunoreactive bands were observed in extracts from cells infected with wild type baculovirus (Fig. 3, lane 1). These results demonstrated that SfManI-GFP and unfused GFP can be expressed by these baculovirus vectors at levels that could be detected by immunoblotting. We must emphasize, however, that the ie1 baculovirus expression vectors used for these experiments typically produce about 50-fold less recombinant protein than a conventional baculovirus expression vector (Jarvis et al., 1996).

3.4. SfManI-GFP resides in punctate structures in the cytoplasm

The intracellular localization of SfManI-GFP was investigated by confocal fluorescence microscopy of live Sf9 cells infected with the baculovirus expression vector encoding

SfManI-GFP. Fluorescence was examined at 18 hours after infection, and Sf9 cells infected with wild type baculovirus or the baculovirus expression vector encoding unfused GFP were used as controls. Cells infected with the baculovirus vector encoding SfManI-GFP contained punctate fluorescent structures dispersed throughout their cytoplasm, but excluded from their nuclei (Fig. 4, top-left panel). In contrast, cells infected with the baculovirus expression vector encoding unfused GFP contained fluorescence throughout their cytoplasm and nuclei, as expected (Fig. 4, top-right panel), and cells infected with wild type baculovirus had no significant fluorescence (Fig. 4, bottom-left panel). If the punctate structures seen in Sf9 cells expressing SfManI-GFP were elements of the Golgi apparatus, then a known Golgi resident enzyme, such as β 1,4-galactosyltransferase (Strous, 1986), should produce a similar pattern of fluorescence. Therefore, we also constructed a baculovirus expression vector encoding a hybrid protein consisting of a bovine β 1,4-galactosyltransferase with GFP fused to its C-terminus (GalT-GFP). Sf9 cells infected with this baculovirus expression vector contained punctate fluorescent structures dispersed throughout their cytoplasm (Fig. 4, bottom-right panel), which was similar to the pattern observed with SfManI-GFP.

3.5. SfManI-GFP colocalizes with a Golgi-specific dye, but not with an ER- or lysosome-specific dye

To further investigate the identity of the punctate structures observed with SfManI-GFP and GalT-GFP, Sf9 cells were infected with the baculovirus expression vectors encoding either one of these two proteins, then labeled with the Golgi-specific, red-fluorescent dye BODIPY-ceramideTR (Fig. 5). The dye produced punctate red-fluorescent structures which significantly overlapped with the green-fluorescent structures produced by GalT-GFP (Fig. 5, top panels). These structures also overlapped with some of the green-fluorescent structures produced by SfManI-GFP (Fig. 5, lower panels). β 1,4-galactosyltransferases usually localize to *trans*-Golgi elements (reviewed in Strous, 1986), while α 1,2-mannosidases, when found in the Golgi, usually localize to *cis* and *medial*-Golgi elements (Velasco et al., 1993). Therefore, it was expected that BODIPY-ceramide, which concentrates in *trans*-Golgi elements, would overlap more extensively with the β 1,4-galactosyltransferase-GFP fusion protein than with the α 1,2-mannosidase-GFP fusion protein.

The cytoplasmic tail of SfManI contains a di-arginine motif (RR) which can serve as an ER-localization signal (Schutze et al., 1994). It also contains a tyrosine-X-X-Ø motif (where X can be any amino acid, and Ø an amino acid with a large hydrophobic side chain), which may direct some proteins to lysosomes (reviewed in Bonifacino et al., 1996). To investigate whether these signals directed any SfManI-GFP to the ER or lysosomes, the fluorescence colocalization assays described above were repeated using ER-(Fig. 6) or lysosome-(Fig. 7) specific dyes. No significant overlap was observed, indicating that neither the potential ER nor the potential lysosomal targeting signal are functional in SfManI-GFP.

4. Discussion

This study was designed to investigate the biosynthesis and intracellular distribution of SfManI. In the first part of this study, we showed that a minor subpopulation of SfManI was N-glycosylated. We also demonstrated that N-glycosylation of SfManI could be dramatically enhanced by changing the original sequon to one that is highly utilized in a mammalian system (Kasturi et al., 1997). Hence, the N-glycosylation efficiency of the wild type and enhanced sequons of SfManI in an insect system correlated with their relative utilization in a mammalian system. Interestingly, when we expressed the catalytic domain of SfManI as a secreted, GST-tagged fusion protein, the vast majority was N-glycosylated at the wild type N-glycosylation sequon of SfManI (Kawar et al., 2000). Therefore, N-glycosylation site usage in SfManI depends not only upon the amino acid composition of the sequon, but also upon the surrounding protein structure.

Apparently, however, N-glycosylation has no significant role in the function of SfManI and other α 1,2-mannosidases. We observed no significant effect on the activity or intracellular localization of SfManI by either eliminating or enhancing its N-glycosylation site (data not shown). Similarly, N-glycosylation of the *Saccharomyces cerevisiae* α 1,2-mannosidase also had no influence on its activity (Herscovics, 1999a). Furthermore, the positions of the sequons are not conserved among various α 1,2-mannosidases (Camirand et al., 1991; Bause et al., 1993; Herscovics et al., 1994; Lal et al., 1994; Tremblay et al., 1998; Kerscher et al., 1995).

In the second part of this study, we investigated the intracellular distribution of SfManI. An SfManI-GFP fusion protein was localized to Golgi elements by confocal fluorescence microscopy. Golgi elements in Sf9 cells appeared as punctate structures located throughout the cytoplasm, in contrast to the perinuclear position of the Golgi in mammalian cells. A similar distribution of the Golgi apparatus has been observed in *Drosophila* cell lines and embryos (Stanley et al., 1997; Rabouille et al., 1999). The ability to observe the Golgi apparatus in live Sf9 cells using SfManI-GFP as a marker will facilitate the study of this integral part of the insect cell secretory system. A better understanding of the secretory system in Sf9 cells is especially important since they are used to produce many secreted proteins via baculovirus expression vectors.

N-glycan processing enzymes are thought to be distributed along the secretory pathway according to the order in which they act, with early-acting enzymes like α -glucosidases being in the ER (reviewed in Moremen et al., 1994) and late acting enzymes like galactosyl-transferases being in *trans*-Golgi elements and the *trans*-Golgi network (reviewed in Strous, 1986). Consistent with this view, α 1,2-mannosidases have been localized to both the ER (Roth et al., 1990; Burke et al., 1996; Bieberich et al., 1997) and Golgi (Velasco et al., 1993; Herscovics et al., 1994; Lal et al., 1994; Bieberich and Bause, 1995), where they act in the intermediate steps of N-glycan processing. It is not yet clear how N-glycan processing enzymes, most of which are type II integral membrane proteins, are targeted to their locations within the secretory pathway. Studies have shown that localization determinants are found in the transmembrane domains and flanking sequences of these enzymes (reviewed in Colley, 1997), as well as in their cytoplasmic tails (Schutze et al., 1994; Slusarewicz et al., 1994; Bryant and Stevens, 1997). On the other hand, a pig α 1,2-mannosidase, which has been localized to the ER (Roth et al., 1990; Bieberich et al., 1997), has cytoplasmic tail and transmembrane domains that are almost identical to those of mouse α 1,2-mannosidase IA, which has been localized to the Golgi (Lal et al., 1994). Further studies of the targeting determinants in α 1,2-mannosidases are needed to address these questions.

Interestingly, the bovine β 1,4-galactosyltransferase was localized in the Golgi apparatus when we expressed it as a GFP-fusion protein in Sf9 cells. This indicates that mammalian Golgi localization signal(s) can function in these insect cells despite the clear difference in the Golgi apparatus morphology.

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Abbreviations

Glc glucose

Man	mannose
GlcNAc	N-acetylglucosamine
ER	endoplasmic reticulum
GFP	green fluorescence protein
PAGE	polyacrylamide gel electrophoresis

References

- Bause E, Bieberich E, Rolfs A, Volker C, Schmidt B. Molecular cloning and primary structure of Man9-mannosidase from human kidney. *European Journal of Biochemistry*. 1993; 217:535–540. [PubMed: 8223597]
- Bieberich E, Bause E. Man9-mannosidase from human kidney is expressed in COS cells as a Golgi-resident type II transmembrane N-glycoprotein. *European Journal of Biochemistry*. 1995; 233:644–649. [PubMed: 7588811]
- Bieberich E, Trembl K, Volker C, Rolfs A, Kalz-Fuller B, Bause E. Man9-mannosidase from pig liver is a type-II membrane protein that resides in the endoplasmic reticulum. cDNA cloning and expression of the enzyme in COS 1 cells. *European Journal of Biochemistry*. 1997; 246:681–689. [PubMed: 9219526]
- Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Analytical Biochemistry*. 1984; 136:175–179. [PubMed: 6424501]
- Bonifacino JS, Marks MS, Ohno H, Kirchhausen T. Mechanisms of signal-mediated protein sorting in the endocytic and secretory pathways. *Proceedings of the Association of American Physicians*. 1996; 108:285–295. [PubMed: 8863342]
- Bryant NJ, Stevens TH. Two separate signals act independently to localize a yeast late Golgi membrane protein through a combination of retrieval and retention. *Journal of Cell Biology*. 1997; 136:287–297. [PubMed: 9015300]
- Burke J, Lipari F, Igdoura S, Herscovics A. The *Saccharomyces cerevisiae* processing alpha 1,2-mannosidase is localized in the endoplasmic reticulum, independently of known retrieval motifs. *European Journal of Cell Biology*. 1996; 70:298–305. [PubMed: 8864657]
- Camirand A, Heysen A, Grondin B, Herscovics A. Glyco-protein biosynthesis in *Saccharomyces cerevisiae*. Isolation and characterization of the gene encoding a specific processing a-mannosidase. *Journal of Biological Chemistry*. 1991; 266:15120–15127. [PubMed: 1714453]
- Cole NB, Smith CL, Sciaky N, Terasaki M, Edidin M, Lippincott-Schwartz J. Diffusional mobility of Golgi proteins in membranes of living cells. *Science*. 1996; 273:797–801. [PubMed: 8670420]
- Colley KJ. Golgi localization of glycosyltransferases: more questions than answers. *Glycobiology*. 1997; 7:1–13. [PubMed: 9061359]
- Deng WP, Nickoloff JA. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analytical Biochemistry*. 1992; 200:81–88. [PubMed: 1595905]
- Herscovics A. Processing glycosidases of *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*. 1999a; 1426:275–285. [PubMed: 9878780]
- Herscovics A. Importance of glycosidases in mammalian glycoprotein biosynthesis. *Biochimica et Biophysica Acta*. 1999b; 1473:96–107. [PubMed: 10580131]
- Herscovics A, Schneikert J, Athanassiadis A, Moremen KW. Isolation of a mouse Golgi mannosidase cDNA, a member of a gene family conserved from yeast to mammals. *Journal of Biological Chemistry*. 1994; 269:9864–9871. [PubMed: 8144579]
- Jarvis DL, Weinkauff C, Guarino LA. Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. *Protein Expression and Purification*. 1996; 8:191–203. [PubMed: 8812860]
- Jarvis DL, Kawar ZS, Hollister JR. Engineering N-glycosylation pathways in the baculovirus–insect cell system. *Current Opinion in Biotechnology*. 1998a; 9:528–533. [PubMed: 9821284]

- Jarvis DL, Wills L, Burow G, Bohlmeier DA. Mutational analysis of the N-linked glycans on *Autographa californica* nucleopolyhedrovirus gp64. *Journal of Virology*. 1998b; 72:9459–9469. [PubMed: 9811679]
- Kasturi L, Chen H, Shakin-Eshleman SH. Regulation of N-linked core glycosylation: use of a site-directed mutagenesis approach to identify Asn–Xaa–Ser/Thr sequons that are poor oligosaccharide acceptors. *Biochemistry Journal*. 1997; 323:415–419.
- Kawar Z, Herscovics A, Jarvis DL. Isolation and characterization of an alpha 1,2-mannosidase cDNA from the lepidopteran insect cell line Sf9. *Glycobiology*. 1997; 7:433–443. [PubMed: 9147053]
- Kawar Z, Romero P, Herscovics A, Jarvis DL. N-glycan processing by a lepidopteran insect α 1,2-mannosidase. *Glycobiology*. 2000; 10:347–355. [PubMed: 10764822]
- Kerscher S, Albert S, Wucherpennig D, Heisenberg M, Schneuwly S. Molecular and genetic analysis of the *Drosophila* mas-1 (mannosidase-1) gene which encodes a glycoprotein processing alpha 1,2-mannosidase. *Developmental Biology*. 1995; 168:613–626. [PubMed: 7729592]
- Kitts PA, Possee RD. A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques*. 1993; 14:810–817. [PubMed: 8512707]
- Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. *Annual Reviews of Biochemistry*. 1985; 54:631–664.
- Lal A, Schutzbach JS, Forsee WT, Neame PJ, Moremen KW. Isolation and expression of murine and rabbit cDNAs encoding an alpha 1,2-mannosidase involved in the processing of asparagine-linked oligosaccharides. *Journal of Biological Chemistry*. 1994; 269:9872–9881. [PubMed: 8144580]
- Marz, L.; Altmann, F.; Staudacher, E.; Kubelka, V. Protein glycosylation in insects. In: Montreuil, J.; Vliegthart, JFG.; Schachter, H., editors. *Glycoproteins*. Elsevier; Amsterdam: 1995. p. 543–563.
- Moremen KW, Trimble RG, Herscovics A. Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology*. 1994; 4:113–125. [PubMed: 8054711]
- Murhammer DW, Gooch CF. Scaleup of insect cell cultures: protective effects of pluronic F-68. *Bio/Technology*. 1988; 6:1411–1418.
- O'Reilly, DR.; Miller, LK.; Luckow, VA. *Baculovirus Expression Vectors*. W.H. Freeman and Company; New York: 1992.
- Rabouille C, Kuntz DA, Lockyer A, Watson R, Signorelli T, Rose DR, van den Heuvel M, Roberts DB. The *Drosophila* GMII gene encodes a Golgi alpha-mannosidase II. *Journal of Cell Science*. 1999; 112:3319–3330. [PubMed: 10504337]
- Roth J, Brada D, Lackie PM, Schweden J, Bause E. Oligosaccharide trimming Man9-mannosidase is a resident ER protein and exhibits a more restricted and local distribution than glucosidase II. *European Journal of Cell Biology*. 1990; 53:131–141. [PubMed: 2076700]
- Schutze MP, Peterson PA, Jackson MR. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO Journal*. 1994; 13:1696–1705. [PubMed: 8157008]
- Sciaky N, Presley J, Smith C, Zaal KJ, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *Journal of Cell Biology*. 1997; 139:1137–1155. [PubMed: 9382862]
- Shaper NL, Shaper JH, Meuth JL, Fox JL, Chang H, Kirsch IR, Hollis GF. Bovine galactosyltransferase: identification of a clone by direct immunological screening of a cDNA expression library. *Proceedings of the National Academy of Science USA*. 1986; 83:1573–1577.
- Slusarewicz P, Nilsson T, Hui N, Watson R, Warren G. Isolation of a matrix that binds medial Golgi enzymes. *Journal of Cell Biology*. 1994; 124:405–413. [PubMed: 8106542]
- Stanley H, Botas J, Malhotra V. The mechanism of Golgi segregation during mitosis is cell type-specific. *Proceedings of the National Academy of Science USA*. 1997; 94:14467–14470.
- Strous GJ. Golgi and secreted galactosyltransferase. *CRC Critical Reviews in Biochemistry*. 1986; 21:119–151. [PubMed: 3093147]
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology*. 1990; 185:60–89. [PubMed: 2199796]
- Summers MD, Smith GE. A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin No 1555*. 1987

- Tremblay LO, Campbell Dyke N, Herscovics A. Molecular cloning, chromosomal mapping and tissue-specific expression of a novel human alpha 1,2-mannosidase gene involved in N-glycan maturation. *Glycobiology*. 1998; 8:585–595. [PubMed: 9592125]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science USA*. 1979; 76:4350–4354.
- Tulsiani DR, Touster O. The purification and characterization of mannosidase IA from rat liver Golgi membranes. *Journal of Biological Chemistry*. 1988; 263:5408–5417. [PubMed: 2965701]
- Vaughn JL, Goodwin RH, Thompkins GJ, McCawley P. The establishment of two insect cell lines from the insect *Spodoptera frugiperda* (Lepidoptera:Noctuidae). *In Vitro*. 1977; 13:213–217. [PubMed: 68913]
- Velasco A, Hendricks L, Moremen KW, Tulsiani DR, Touster O, Farquhar MG. Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. *Journal of Cell Biology*. 1993; 122:39–51. [PubMed: 8314846]
- Webb NR, Summers MD. Expression of proteins using recombinant baculoviruses. *Technique*. 1990; 2:173–188.

Fig. 1.

Glycosylation of SfManI. Sf9 cells were infected with a baculovirus expression vector encoding SfManI, labeled with tran-³⁵S-label, and total intracellular proteins were extracted at 28 h after infection. Some cultures were treated with 1.5 μ g of tunicamycin per ml of medium (lanes 3 and 4). SfManI was immunoprecipitated from the extracts with an anti-SfManI antiserum, and immunoprecipitates were treated with either EndoH_f (lanes 2 and 4) or buffer alone (lanes 1 and 3). The samples were then analyzed by SDS-PAGE and autoradiography. The positions and sizes (kDa) of protein markers are shown on the left-hand side of the figure and the position of SfManI is shown on the right.

Fig. 2.

Analysis of SfManI N-glycosylation mutants. Sf9 cells were infected with baculovirus vectors encoding wild type SfManI (WT), the knockout mutant (Δ N), or the enhanced N-glycosylation mutant (eN). The cells were labeled with tran-³⁵S-label and total intracellular proteins were extracted at 28 h after infection. SfManI was immunoprecipitated from the extracts with an anti-SfManI antiserum and immunoprecipitates were treated with either EndoH_F (+) or buffer alone (-). The samples were then analyzed by SDS-PAGE and autoradiography. The positions and sizes (kDa) of protein markers are shown on the left-hand side of the figure and the position of SfManI is shown on the right.

Fig. 3.

Expression of SfManI-GFP and unfused GFP. Sf9 cells were infected with wild type baculovirus (lane 1), a baculovirus vector encoding unfused GFP (lane 2), or a baculovirus vector encoding SfManI-GFP (lane 3). Total intracellular protein extracts were prepared at 24 h post infection and analyzed by SDS-PAGE and immunoblotting with a polyclonal antiserum against GFP. The positions and sizes (kDa) of protein markers are shown on the left-hand side of the figure and the positions of GFP and SfManI-GFP are shown on the right.

Fig. 4.

Intracellular distribution of SfManI-GFP. Sf9 cells were infected with wild type baculovirus or baculovirus vectors encoding SfManI-GFP, unfused GFP, or GalT-GFP. Unfixed cells were examined by confocal fluorescence microscopy 18 h after infection.

Fig. 5.

Colocalization of SfManI-GFP with a Golgi-specific dye. Sf9 cells were infected with baculovirus vectors encoding either SfManI-GFP (bottom panels) or GalT-GFP (top panels). At 18 h post infection, the cells were labeled with BODIPY TR ceramide, and unfixed cells were examined by confocal fluorescence microscopy. The panels on the left represent the fluorescence pattern of the GFP fusion proteins. The panels on the right represent the fluorescence pattern of the dye. The panels in the middle represent an overlay of the panels on either side.

Fig. 6.

Lack of colocalization of SfManI-GFP with an ER-specific dye. Sf9 cells were infected with baculovirus vectors encoding either SfManI-GFP (bottom panels) or GalT-GFP (top panels). At 18 hours post infection, the cells were labeled with DiIC₆₍₃₎, and unfixed cells were examined by confocal fluorescence microscopy. The panels on the left represent the fluorescence pattern of the GFP fusion proteins. The panels on the right represent the fluorescence pattern of the dye. The panels in the middle represent an overlay of the panels on either side.

Fig. 7.

Lack of colocalization of SfManI-GFP with a lysosome-specific dye. Sf9 cells were infected with baculovirus vectors encoding either SfManI-GFP (bottom panels) or GaIT-GFP (top panels). At 18 h post infection, the cells were labeled with LysoTracker Red DND-99, and unfixed cells were examined by confocal fluorescence microscopy. The panels on the left represent the fluorescence pattern of the GFP fusion proteins. The panels on the right represent the fluorescence pattern of the dye. The panels in the middle represent an overlay of the panels on either side.