Co-translational Excision of α-Glucose and α-Mannose in Nascent Vesicular Stomatitis Virus G Protein

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ABSTRACT Membrane bound polysomes were prepared from HeLa cells infected with vesicular stomatitis virus (VSV), after pulse labeling with [^{3}H]mannose for various times from 15 to 90 min. Oligosaccharides on nascent chains were released from peptides by treatment with endoglycosidase H and sized by high resolution Biogel P4 chromatography. Processing on some nascent chains proceeded to the removal of all three types of α-linked glucose and one α-1,2-mannose from the $Glc_3Man_9GlcNAc$ precursor showing that the enzymes responsible were not only active on nascent chains but were present in the rough endoplasmic reticulum (RER). Incubation of cells for various times in cycloheximide, where chain elongation had ceased, made no difference to the profile of oligosaccharides on the nascent chains, and trimming proceeded no further than $Man_8GlcNAc_2Asn$. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an energy inhibitor reportedly able to block the transfer of glycoproteins from the RER, increases the amount of Man_8-oligosaccharides on the nascent chains and also the amount of $Glc_3Man_9GlcNAc$ precursor. On completed G protein in the RER fraction from which membrane bound polysomes were prepared, processing occurred to Man_6- but not to $Man_5GlcNAc$ sized oligosaccharides in the CCCP-treated cells. By contrast, processing to $Man_5GlcNAc$ oligosaccharides was observed in unfractionated control cells.

The biosynthesis and insertion of vesicular stomatitis virus (VSV)[1] G protein into membrane is a widely studied model system (reviewed by Rothman and Lenard [26]) for probing the membrane assembly behavior of N-linked glycoproteins. The molecule contains two glycosylation sites (25, 28) that initially receive a $Glc_3Man_9GlcNAc_2$ or "high mannose" precursor (15, 16, 24). A number of laboratories have elucidated in detail (reviewed by Hubbard and Ivatt [14]) the subsequent carbohydrate processing or "trimming" reactions, in this and many other glycoproteins, involving α-glucosidase and α-mannosidase action before the maturation of the oligosaccharide structure to its complex form (18, 23) by the action of *N*-acetylglucosaminyl transferases, galactosyl transferases, and fucosyl and sialyl transferases. By the criteria of cell-free synthesis and glycosylation of G protein in vitro, the initial glycosylation is a co-translational event (17, 27). While there is direct evidence that N-linked glycosylation occurs co-translationally in vivo (2), the form of glycosylation and extent of processing on nascent chains has not yet been examined in any system.

Glucosidase II releases α-1,3 linked glucoses from $Glc_2Man_9GlcNAc_2$ and has been purified from rat liver endoplasmic reticulum (6) but has not been specifically located to the rough endoplasmic reticulum (RER). Mannosidase II cleaves α-1,6-linked and α-1,3-linked mannose and has been identified in a Golgi membrane preparation from rat liver as has mannosidase IA (30) and IB (34), the enzymes responsible for cleaving α-1,2 linked mannoses. There are four α-1,2 linked mannoses in the $Glc_3Man_9GlcNAc_2$ precursor, and it is conceivable that there are more than two α-1,2-mannosidase activities, since yeast has only one α-1,2-mannosidase that cleaves one specific α-1,2 mannose residue out of three such terminal mannoses in the same precursor (7). An α-1,2-mannosidase activity has been demonstrated in the RER of bovine thyroid cells (11). These activities are evident in the RER in other circumstances. For example, human Z-variant α_1-antitrypsin accumulates in the RER and the high mannose oligosaccharides were found to be Man_7-, Man_6-, and $Man_5GlcNAc$ (13). Influenza virus HA is blocked in the RER in carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) treated cells, and it has been shown that mannoses are trimmed from hemagglutinin high mannose oligosaccharides under these conditions (8). Sindbis virus B protein, a RER precursor of the virus structural glycoproteins, has high mannose oligosaccharides which can be trimmed to $Man_8GlcNAc_2$ (12). More recently an α-1,2-mannosidase has been identified

[1] *Abbreviations used in this paper:* CCCP, carbonyl cyanide m-chlorophenylhydrazone; RER, rough endoplasmic reticulum; VSV, vesicular stomatitis virus.

in a preparation of RER of rat liver, which removed α-mannose from Man_9-, Man_8-, Man_7-, and Man_6- compounds, but not from $Man_5GlcNAc_2Asn$ (3).

Direct assignment of enzyme activities to a specific subcellular compartment based on purification of membranous fractions is usually weakened by questions of the degree of cross contamination by membranes from different compartments. We therefore decided to examine the degree of processing on nascent chain VSV G glycoprotein to circumvent the problem and determine what processing occurs in the RER in this widely studied model system. We have found high mannose forms down to $Man_8GlcNAc$ are present on nascent chains implying all glucosidases active in processing $Glc_3Man_9GlcNAc_2$ can act co-translationally, and that α-1,2-mannosidase acts co-translationally.

MATERIALS AND METHODS

Cells, Virus, Infection and Labeling: HeLa S3 cells grown in suspension culture were concentrated and infected with 20 pfu/cell of VSV (1). Infection was allowed to proceed for 4.5 h and then these cells were labeled with 20–40 μCi/ml 2-[^{3}H]mannose (New England Nuclear, Boston, MA). At specified times thereafter, cultures were divided for the addition of cycloheximide (15 μg/ml), valinomycin (Sigma Chemical Co., St. Louis, MO, 5 μM) or CCCP (Sigma Chemical Co., 80 μg/ml) and labeling was continued as noted in the text. Membrane bound and free polysomes were prepared as previously described (1). Essentially, a microsomal pellet was obtained by differential centrifugation of the postnuclear supernatant. RER was further purified from this by a rate-zonal sedimentation through a 15–30% sucrose gradient, then resuspended in buffer containing 1% sodium deoxycholate. This material was then centrifuged on a 7–52% sucrose gradient to separate polysomes from solubilized materials. Labeled oligosaccharides were never found associated with the free polysome fraction.

Enzyme Digestions and Column Chromatography: Pooled fractions from polysome gradients were lyophilized, resuspended in 1.2 ml 100 mM Tris, pH 8, 10 mM $CaCl_2$, 1.2 mg/ml pronase, and digested at 37°C for 24 h. An additional 1.2 mg/ml pronase was added and the digestion continued another 24 h. [^{14}C]Mannose labeled Sindbis virus glycopeptides were added as markers. The glycopeptides were digested with 0.02 U/ml of endo-β-*N*-acetylglucosaminidase-H (purified in this laboratory as previously described [12]) in 0.05 M citrate-phosphate buffer, pH 5.0 at 37°C for 12 h. The released oligosaccharides as well as undigested glycopeptides were separated into component size classes by high resolution Biogel P4 gel filtration chromatography, as previously described (12). Jack bean α-mannosidase was purified and used as previously described (20).

RESULTS

Nascent Chains

To examine the glycosylation on nascent chains of VSV G protein, membrane-bound polysomes were prepared from infected HeLa cells labeled with [^{3}H]mannose. PAGE with fluorography confirmed that G protein was the only labeled glycoprotein in the infected cells (data not shown). Polysomes were released from membranes by detergent treatment and then separated on a sucrose gradient (Fig. 1*A*). [^{3}H]Mannose-labeled glycopeptides from these G protein nascent chains were prepared by Sephadex G-25 column chromatography, after extensive pronase digestion. At this stage in the preparation of the oligosaccharides, there was not sufficient radioactivity incorporated to follow glycopeptide elution position by assaying [^{3}H]mannose radioactivity, and glycopeptides were pooled by the expected chromatographic behavior on a calibrated column. High mannose oligosaccharides were released by endoglycosidase-H digestion and the products separated by chromatography on high resolution Biogel P4 columns. In the 60-min-labeled cells, oligosaccharides present were of the size $Glc_3Man_9GlcNAc$, $Glc_2Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$, and a shoulder of

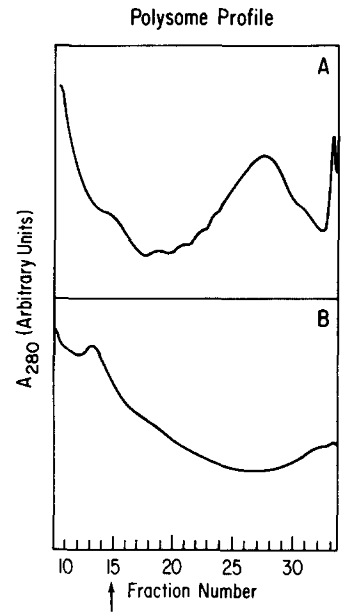

FIGURE 1 Preparation of membrane bound polysomes. HeLa cells, 4 × 10^7 in 100 ml, were infected for 4.5 h with vesicular stomatitis virus (20 pfu/cell), concentrated twofold, labeled 15 min with 40 μCi/ml 2-[^{3}H]mannose and the culture split into two. One half was maintained as a control while CCCP was added to the other half to a final concentration of 80 μg/ml. Labeling continued for the next 45 min and then the membrane bound polysomes were prepared. Gradients were fractionated and the absorbancy profiles recorded. (*A*) Membrane bound polysomes from untreated cells. Fractions 23–33 were collected and pooled. (*B*) Membrane bound polysomes from CCCP-treated cells; fractions 16–30 were collected and pooled. The arrow shows the position of ribosomal 60s subunits.

$Man_8GlcNAc$ as determined by cochromatography with authentic [^{14}C]mannose labeled Sindbis virus oligosaccharides of these compositions (Fig. 2*A*). We concluded that processing with glucosidases was occurring on the nascent chains, and the small but definite amount of $Man_8GlcNAc$ sized oligosaccharides present on nascent chains showed that an α-1,2-mannosidase was acting co-translationally as well.

Cycloheximide and CCCP Treatment

To test whether further mannosidase processing occurred on nascent chains "frozen" in translation, infected cells were pulse labeled for 30 min with [^{3}H]mannose and then incubated a further 30 min in cycloheximide containing labeled medium. The oligosaccharide profile from nascent chains was virtually identical to that shown in untreated cells (see Fig. 2*A*) and to control cells labeled 60 min in the absence of cycloheximide (data not shown). There was no further trimming of mannoses from nascent chains frozen during translation.

We decided to observe the effects on processing of G-protein oligosaccharides in cells blocked in translocation from the RER. To limit newly completed G protein to the RER, cells were pulse-labeled for 15 min with [^{3}H]mannose and then incubated a further 45 min in the presence of CCCP, a reported inhibitor of translocation of proteins from the RER to the Golgi apparatus (30). Control cultures were pulsed for 60 min with [^{3}H]mannose only. CCCP treatment caused a marked flattening of the polysome profile (Fig. 1*B*), and equivalent portions from the polysome regions of the control and drug treatment gradient were pooled. In contrast to the control cells, G-protein nascent chains from cultures treated with CCCP showed a marked build up in the $Man_8GlcNAc$ sized species (Fig. 2*B*) as well as a large increase in the amount of $Glc_3Man_9GlcNAc$ sized oligosaccharide. In cultures treated with valinomycin, another type of energy inhibitor, in the same labeling regimen as that described above for CCCP, there was no effect on the processing of nascent chains or in the newly synthesized G protein found in the RER fraction.

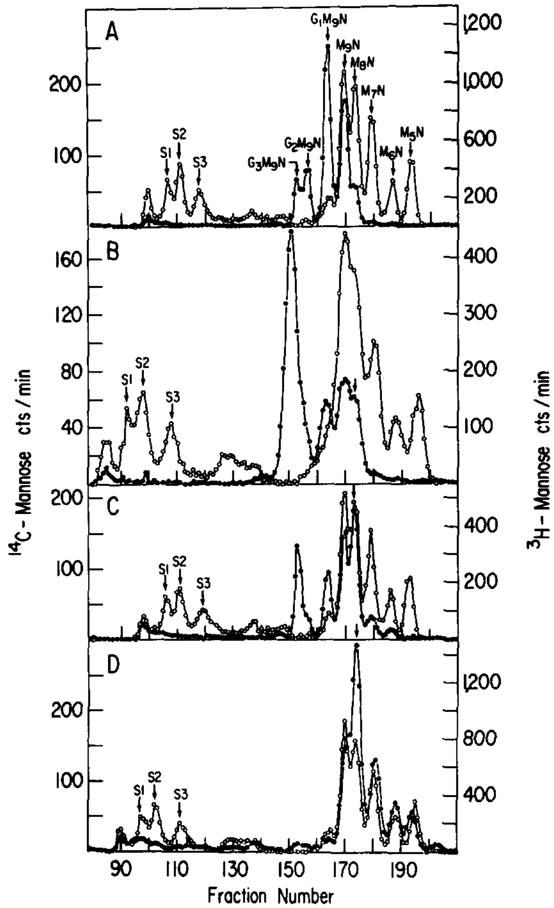

FIGURE 2 Biogel P4 chromatographic separation of VSV-G protein oligosaccharides. Nascent chains from membrane bound polysomes, labeled with 2-[^{3}H]mannose, were digested exhaustively with pronase and the glycopeptides purified by Sephadex G-25 chromatography. High-mannose oligosaccharides were released by digestion with endo-β-*N*-acetylglucosaminidase-H and separated by high resolution Biogel P4 chromatography (−400 mesh, 1.5 × 175 cm). (*A*) Nascent chain oligosaccharides labeled for 60 min with 2-[^{3}H]mannose (●). [^{14}C]Mannose labeled Sindbis virus markers (○). In this and other panels the left-most eluting peak is insoluble material eluting with the void volume of the column. (*B*) Nascent chain oligosaccharides labeled 15 min with 2-[^{3}H]mannose (●), then a further 45 min in the presence of CCCP. Sindbis virus marker oligosaccharides labeled with [^{14}C]mannose (○). (*C*) Oligosaccharides of completed G protein, released from rough endoplasmic reticulum by 1% sodium deoxycholate. Oligosaccharides were labeled 15 min with 2-[^{3}H]mannose, then a further 45 min in the presence of CCCP (●). Sindbis virus, marker oligosaccharides labeled with [^{14}C]mannose (○). (*D*) Oligosaccharides of completed G protein in the unfractionated whole cells, labeled for 60 min with 2-[^{3}H]mannose (●). [^{14}C]Mannose labeled Sindbis virus oligosaccharides (○). Note the presence of the more processed oligosaccharides $Man_5GlcNAc$ and significant accumulations of S1 and S2 complex oligosaccharides (fractions 95–105).

Other labeling regimens were used, such as a 20-min pulse with a further 70 min in CCCP, and the results were essentially identical.

Oligosaccharide Sizes in Fractions

Newly synthesized G protein found in RER, is released by detergent treatment and is found in the load zone of the sucrose gradient used to prepare polysomes. The distinctness of the nascent chain fraction (Fig. 2, *A* and *B*), from the fraction containing the newly completed G protein from the RER in CCCP-treated cells (Fig. 2*C*) and the G protein in whole untreated cells (Fig. 2*D*), is evident from lack of Man_7-GlcNAc and $Man_6GlcNAc$ in the nascent chains (Fig. 2*A*) compared to the RER G protein. Likewise not all glucoses were removed from G protein in the RER fractions prepared as described here, whereas this removal was relatively complete in the accumulated G protein in the whole cells (Figs. 2, *A*, *B*, and *C*; cf. Fig. 2*D*). There was also a lack of $Man_5GlcNAc$ in the RER compartment (Fig. 2*C*) compared to the unfractionated whole cells (Fig. 2*D*). Essentially the same profile (Fig. 2*C*) was obtained in RER of cells labeled 60 min but not treated with CCCP (data not shown) and hence the drug did not cause any noticeable accumulation of Man_5 oligosaccharides in this fraction. In other experiments, cells were labeled for 30 or 90 min with [^{3}H]mannose and the oligosaccharide profile of G protein in the RER fraction examined. Trimming again progressed to $Man_6GlcNAc_2Asn$ but no further (data not shown) with a relative build up in $Man_8GlcNAc$ oligosaccharides. We conclude the activity generating $Man_5GlcNAc_2Asn$ from Man_6- is not present in the crude rough endoplasmic reticulum fraction as prepared here. Unfractionated cells also predominated in $Man_8GlcNAc$ but contained, in addition, significant amounts of Man_7-, Man_6-, and $Man_5GlcNAc$ (Fig. 2*D*). There were no complex sialic acid or galactose containing glycopeptides present (see the S1, S2, S3 region, Fig. 2, *A*, *B*, and *C*) in any of labeling conditions used in the nascent chains or in the crude RER fraction, though some accumulation in S1, S2, and S3 were observed in the unfractionated cells (Fig. 2*D*). Compared to the high mannose glycopeptides, G-protein–containing complex oligosaccharides did not appear to accumulate intracellularly. This may reflect a relatively quick turnover of this pool as completed G protein leaves the cells (1).

Mannosidase Treatment of Oligosaccharides

To determine whether there was significant $Man_8GlcNAc_2$-P-P-dol giving rise to $Man_8GlcNAc_2Asn$ on nascent chains, selected precursor oligosaccharides were digested with jack bean α-mannosidase. The rationale for doing so derives from evidence (9) that shows truncated precursors are processed, so far as removal of glucoses are concerned, in a fashion similar to the usual Glc_3Man_9- precursor. Thus, a Glc_3Man_8- presursor should also give rise to Glc_2Man_8- and Glc_1Man_8- precursors. These would expectedly be found in the size range occupied by Glc_1Man_9- and Man_9- on Biogel P4 columns. Similar considerations apply to possible Glc_3Man_7- precursors. Therefore, Glc_1Man_9-, Man_9- and also Man_8- sized peaks were isolated (Fig. 3*A*). Rechromatography of the isolated Man_8- compound pooled typically as the others showed it to be homogeneous (data not shown). Isolated compounds were then digested with jack bean α-mannosidase. When the $Glc_1Man_9GlcNAc$ peak was digested, a product the size of $Man_{5.5}GlcNAc$ was detected (Fig. 3*B*) as would be expected for a high mannose compound containing terminal glucoses, in this case of composition $Glc_1Man_4GlcNAc$ (31). There was also a small amount of $Man_{6.5}GlcNAc$. This was also expected because of the comparative resistance to jack bean α-mannosidase of the Man-α-1,6 attached to the core β-1,4-mannose in these compounds (32). $Man_9GlcNAc$ from completed VSV G protein in the crude RER fraction of CCCP-treated cells yielded products the size of Man-β-GlcNAc and free mannose in the ratio of 1:7.6 (Fig. 3*C*). There were no other significant products (Fig. 3*B*), especially in the size range of Glc_1Man_4-

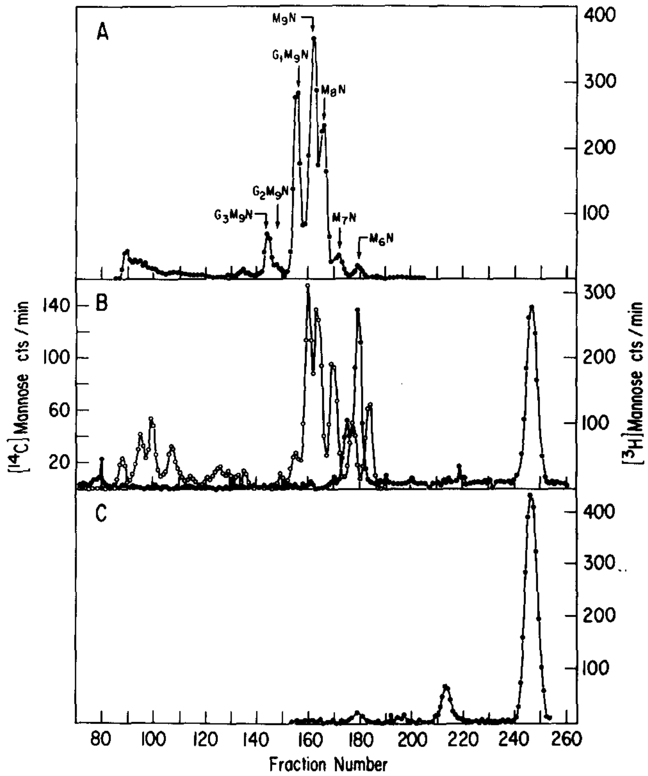

FIGURE 3 Biogel P4 chromatography of mannosidase digested oligosaccharides. Glycopeptides were purified, digested with endoglycosidase-H, and oligosaccharides separated by high resolution Biogel P4 chromatography. (*A*) Total oligosaccharides, labeled for 60 min with 2-[^{3}H]mannose (●), from completed G protein, released from crude rough endoplasmic reticulum by 1% sodium deoxycholate. (*B*) The peak corresponding to Glc_1Man_9GlNAc in the chromatography depicted in the previous panel was pooled and isolated. 2-[^{3}H]Mannose labeled oligosaccharides (●) were further digested with 10.6 units of milliliter jack bean α-mannosidase for 48–96 h at 37°C and the products analyzed by high resolution Biogel P4 chromatography. [^{14}C]Mannose labeled Sindbis virus marker oligosaccharides (○). (*C*) Same as in *B*, except the $Man_9GlcNAc$ peak, labeled with 2-[^{3}H]mannose, (●) was purified and digested with mannosidase before further analysis by Biogel P4 chromatography.

GlcNAc (31) that might have been expected if the Man_9 peak contained significant amounts of $Glc_1Man_8GlcNAc$. When $Man_8GlcNAc$ was digested with jack bean α-mannosidase, the products were an oligosaccharide the size of Man-β-GlcNAc and free mannose in the ratio of 1:7.2.

These digestions with α-mannosidase show that oligosaccharides that appear in the nascent chain profile, of the size $Man_8GlcNAc$, could not have arisen from a glucose containing $Man_8GlcNAc$ species originally present in the dol-P-P-oligosaccharide precursor.

DISCUSSION

We have shown that processing of the $Glc_3Man_9GlcNAc_2Asn$ precursor on VSV G protein occurs on nascent chains allowing us to conclude that glucosidases and an α-1,2-mannosidase act co-translationally. The possibility that these activities were contributed by membranes from sources other than RER was obviated because the polysomes sedimented by virtue of their size (numbers of ribosomes on the mRNA) after detergent dissolution of the membranes and hence membrane bound glycoprotein would not be found in this region of the gradient. These results confirm and extend previous observations of an α-1,2-mannosidase activity in the rough endoplasmic reticulum (11, 13) because we have now shown that one such activity occurs on nascent chains of VSV G protein and proceeds only as far as formation of $Man_8GlcNAc_2Asn$. Such RER α-mannosidase activity was also evident in the trimming of Sindbis virus B protein oligosaccharides (12), which showed the presence of $Man_8GlcNAc_2Asn$, the product of this mannosidase cleavage. In collaboration with others, we have analyzed the extent of trimming of N-Asn linked oligosaccharides in several other rough endoplasmic reticulum proteins. For example, ribophorins are located in the RER (19) and when labeled in a one hour pulse with [^{3}H]mannose, ribophorin I also shows trimming to $Man_8GlcNAc_2Asn$ (Rosenfeld, M. G., E. E. Marcantonio, J. Hakimi, V. H. Ort, P. H. Atkinson, D. D. Sabatini, and G. Kreibich, manuscript in preparation). Simian rotavirus SA11 specific glycoproteins VP7 (22) and probably also NCVP5 are located in the RER and show trimming to Man_8 (4, 5, 16), again providing evidence of the RER location of this α-1,2-mannosidase. α-Mannosidase II is responsible for trimming α-1,3- and α-1,6-linked mannose residues from the precursor (29, 33) and has recently been shown by immunoelectron microscopy (21) to be located predominantly in the Golgi apparatus with some of the reaction product visible in the RER. The relationship between the α-mannosidase IA and IB activities (29, 33), the RER α-mannosidase activity (3) and the nascent chain α-mannosidase activity remains to be demonstrated. It is clear, however, that mannoses are removed from G protein in the RER. This observation differs with a recent study of VSV G protein in Chinese hamster ovary cells in which it was concluded all mannoses are removed in the Golgi apparatus (10). Our evidence shows trimming of such RER G protein oligosaccharides to $Man_6GlcNAc_2Asn$ but not to $Man_5GlcNAc_2$-Asn. The RER fraction containing these processed G-protein oligosaccharides was not highly purified; however, it was clearly sufficiently so to distinguish it from unfractionated whole cells where $Man_5GlcNAc_2Asn$-sized oligosaccharides on G-protein were observed. Trimming only as far as Man_6- in the oligosaccharides on completed glycoproteins in RER does not seem to be a general observation because oligosaccharides on other proteins clearly are processed further (13, 16) in this compartment. Though all three glucoses could be removed from G protein in the RER, this was not complete, and only the accumulated molecules in the unfractionated cells showed complete removal of glucoses. Whether glucoses are completely removed just before transit from the RER or whether this occurs in a later compartment for this protein remains to be determined.

How blocking G protein in the RER with CCCP affects glucosidase I and the Man_8 to Man_7 trimming enzymes (Fig. 2*B*) is not at all clear. It is probable the drug affects energy generation in the cells and this indirectly affects processing even though these reactions are not energy-dependent. Whether the α-1,2-mannosidase active on nascent chains would cleave a specific α-1,2-mannose, out of four possible, as shown in our recent studies of yeast invertase trimming (7) also remains to be demonstrated.

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