Glucosidase I, a Transmembrane Endoplasmic Reticular Glycoprotein with a Luminal Catalytic Domain*

(Received for publication, October 10, 1990)

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We have analyzed the functional domain structure of rat mammary glucosidase I, an enzyme involved in N-linked glycoprotein processing, using biochemical and immunological approaches. The enzyme contains a high mannose type sugar chain that can be cleaved by endo-β-N-acetyl-d-glucosaminidase H without significantly affecting the catalytic activity. Based on trypsin digestion pattern and the data on membrane topography, glucosidase I constitutes a single polypeptide chain of 85 kDa with two contiguous domains: a membrane-bound domain that anchors the protein to the endoplasmic reticulum and a luminal domain. A catalytically active 39-kDa domain could be released from membranes by limited proteolysis of saponin-permeabilized membranes with trypsin. This domain appeared to contain the active site of the enzyme and had the ability to bind to glucosidase I-specific affinity gel. Phase partitioning with Triton X-114 indicated the amphiphilic nature of the native enzyme, consistent with its location as an integral membrane protein, whereas the 39-kDa fragment partitioned in the aqueous phase, a characteristic of soluble polypeptide. These results indicate that glucosidase I is a transmembrane protein with a luminally oriented catalytic domain. Such an orientation of the catalytic domain may facilitate the sequential processing of asparagine-linked oligosaccharide, soon after its transfer en bloc by the oligosaccharide transferase complex in the luminal domain of endoplasmic reticulum.

The biosynthesis of N-linked glycoproteins involves the formation of a lipid-linked oligosaccharide precursor, Glc₃Man₉GlcNAc₂-P-P-dolichol. An en bloc transfer of the tetradecasaccharide to the nascent polypeptide in the rough endoplasmic reticulum (ER) is followed by a series of processing reactions. These reactions involve the excision of all the glucosyl residues; depending upon individual glycoprotein, one or more mannose residues may be removed and additional sugars may be added as the glycoprotein reaches its final destination, either within a membrane compartment of the cell or secretion into the extracellular environment. The processing may begin as soon as the glycosylation of the appropriate asparagine residue has occurred while the polypeptide backbone is still undergoing synthesis on the poly- somes. These reactions continue as the newly assembled glycoprotein is being transported from the rough ER through the Golgi compartment during the terminal stages of its assembly.

The ensemble of glycosyltransferases and glycosidases involved in oligosaccharide assembly and processing act sequentially and in concert in which the product of one enzyme serves as the substrate for the next (3–5). Studies on membrane topography indicate that the biosynthesis of intermediates up to Man₃GlcNAc₂-P-P-dolichol occurs on the cytoplasmic face, whereas the transfer of additional mannose and glucosyl residues takes place in the lumen of the ER (6). The first enzyme of the dolichol cycle, viz. UDP-GlcNAcdolichol-P GlcNAc-1-P-transferase has been purified to homogeneity (7) and the membrane orientation of the enzyme, postulated from the cDNA sequence (8), is in agreement with the proposal that the catalytic domain of the enzyme is oriented toward the cytoplasmic face of ER membrane. Elongation of Man₃GlcNAc₂-P-P-dolichol to the ultimate precursor, Glc₃Man₉GlcNAc₂-P-P-dolichol, has been proposed to occur within the lumen of ER. Indeed, the catalytic domain of GDP-Man:dolichol-P mannosyltransferase, the enzyme that generates Man-P-dolichol for the elongation reactions, appears to be oriented toward the lumen (9, 10).

Among the processing-specific glycosidases located in the rough ER, glucosidases I and II cleave the α-1,2- and α-1,3-linked glucosyl residues on the precursor (2, 11, 12). The kinetic pattern of the removal of glucosyl residues indicated that the innermost glucosyl residue is removed at a later time point than the outer 2 residues, suggesting the location of glucosidases at different places along the secretory pathway (13). Glucosidase II has been localized in smooth and rough ER through immunoelectron microscopy (14, 15). Besides the glucosyl residues, one of the mannose residues in the oligosaccharide moiety is also removed by an α-mannosidase in rough ER (16).

Although the precise molecular basis for the localization of the above glycosidases has not been elucidated, current views favor the idea that the residence of a particular protein in an organelle requires a specific retention signal that retains the protein in that location. Such retention sequences have been identified in both soluble (17) and transmembrane (18, 19) ER resident proteins. For example, ER residence of the adenoviral E3/19K protein, a type I transmembrane protein, requires the last six amino acids (DEKKMP) at the C-
terminal end of the short cytoplasmic tail (20). It is possible that the retention of processing glycosidases in ER could be determined by such sequences in their cytoplasmic tail.

Previously, we reported the purification of glucosidase I from rat mammary gland (11), the enzyme activity was shown to be modulated as a function of gland ontogeny (21). It was also observed that the hormonal regulation of the enzyme activity in explant cultures was in good agreement with the glycosylation of α-lactalbumin, an N-linked glycoprotein and a characteristic marker of the lactating tissue (22). Given the positioning of glucosidase I in the multi-enzyme pathway of oligosaccharide assembly and processing, it is likely that this enzyme may play a critical role in the overall biosynthesis and regulation of protein N-glycosylation. In this investigation, we report the functional organization of glucosidase I in the ER membranes. Our results show that it is a transmembrane glycoprotein with a short cytoplasmic tail. The implications of distinct domains of the enzyme vis-à-vis its retention in ER also are discussed.

MATERIALS AND METHODS

All chemicals and reagents employed in the purification of glucosidase I and preparation of anti-rabbit antibodies against the enzyme were obtained from commercial sources (11). Sprague-Dawley lactating female rats were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA). 1-Deoxynojirimycin was a kind gift from Drs. D. Schmidt and Scangos, Bayer AG, Wuppertal, Federal Republic of Germany. Enzymes endo H, endo F, and N-glycnase were obtained from Genzyme (Cambridge, MA). Biotinylated lectins and alkaline-phosphatase substrate kit were purchased from Vector Laboratories (Burlingame, CA). Anti-rabbit IgG and modified trypsin were products of Promega Biotech (Madison, WI). Anti-rabbit [125I]IgG and Rainbow molecular weight markers were products of Amersham Corp. All other analytical grade fine chemicals were purchased from Sigma.

Purification of Glucosidase I and Preparation of Antibodies—The purification of glucosidase I was carried out essentially as described (11). The purified enzyme was stored in 200 mM potassium phosphate buffer, pH 6.8, containing 0.5% Lubrol PX (buffer A) at -70 °C. Anti-glucosidase I antibodies were raised in rabbit and affinity-purified by the methods described (7, 23).

Glucosidase I Assay—Glucosidase I assay was carried out by incubating the crude or pure enzyme preparation with 10,000 cpm of [14C]GlcMAN GlcNAC in 50 mM potassium phosphate buffer containing 0.25% Lubrol PX in a final reaction volume of 100 μl. The reaction mixtures were incubated for 30 min at 37 °C, and the reaction was stopped by boiling for 2 min. The release of [14C]Glc was determined by such sequences in their cytoplasmic tail.

Results

Characterization of Glucosidase I—Although, the purified glucosidase I exhibited a band of 85 kDa on 10% SDS-PAGE under reducing conditions (21), it was resolved into two bands of 82 and 85 kDa on 12.5% SDS-PAGE in Tricine buffer system (24) (Fig. 1a). The purified glucosidase I from calf liver exhibited a doublet of about 85 kDa on 10% SDS-PAGE (32). Peptide mapping of the two bands by chemical cleavage with N-chlorosuccinimide revealed identical fragments indicating similarities in their primary structures (Fig. 1b). Con A-Sepharose binding suggested that glucosidase I from rat mammary gland is an N-linked glycoprotein. To examine the nature of its sugar moiety, the purified enzyme was digested with endo H, endo F, and N-glycnase. In each case, the digestion resulted in an increased electrophoretic mobility (decrease in molecular mass of approximately 2000 Da) of

Membrane Topography of Glucosidase I

Peptide Mapping—5 μg of pure glucosidase I was subjected to 12.5% SDS-PAGE in Tricine buffer system (24) for 16 h. After staining and destaining, the gel portions corresponding to bands A and B were cut out separately with a razor blade. The peptide mapping of the polypeptide bands was carried out by chemical cleavage with 0.015 M N-chlorosuccinimide in urea:acetic acid:water (1 g:l ml:l ml), buffer, pH 6.8, containing 0.8% Lubrol PX (buffer A) at -70 °C.

Endo Enzyme Digestions—The digestion resulted in an increased electrophoretic mobility (decrease in molecular mass of approximately 2000 Da) of
both polypeptides (Fig. 1), indicating the removal of one high mannose type N-linked oligosaccharide. Biotinylated Con A did not bind either of the polypeptide bands following digestion with endo enzymes, confirming that the sugar moiety was cleaved (Fig. 2B). Next, the purified enzyme was incubated at 37 °C with or without endo H for different time intervals, and its activity was determined. A complete deglycosylation of glucosidase I could be achieved within 1–2 h of incubation (Fig. 3); at this time point, the catalytic activity of the deglycosylated enzyme was comparable with that of the native enzyme. Further incubation under these conditions resulted in a rapid decline in the activity of the enzyme in both control as well as experimental sets, possibly due to enzyme inactivation at 37 °C. Nevertheless, the catalytically active enzyme after digestion with endo H did not bind Con A-Sepharose (data not shown).

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**FIG. 1.** a, 5 μg of purified glucosidase I from rat mammary gland was subjected to 12.5% Tricine-SDS-PAGE. After staining with Coomassie Brilliant Blue and destaining with 50% methanol containing 10% acetic acid, protein bands A and B were cut out carefully with a razor blade. The gel pieces containing bands A and B were used for peptide mapping by chemical cleavage with N-chlorosuccinimide (25). b, lane 1, band A, and lane 2, band B.

**FIG. 2.** Purified glucosidase I was digested with endo H (lane 2), endo F (lane 4), and N-glycnase (lane 6). Controls for each endo enzyme were carried out under identical conditions except that the endo enzyme was omitted from the reaction mixture (lanes 1, 3, and 5). Following digestion, samples were boiled with Laemmli's buffer and subjected to electrophoresis on 10% SDS-PAGE followed by electrotransfer to nitrocellulose membrane. A, Western blotting with anti-glucosidase I antibodies; B, biotinylated Con A-binding assays.

**FIG. 3.** Effect of deglycosylation on enzyme activity. Pure glucosidase I was incubated with endo H at 37 °C. At the indicated time interval, samples were withdrawn and a portion was used immediately for the determination of glucosidase I activity. Controls were run under identical conditions except that endo H was omitted from the reaction mixtures. The remaining sample was subjected to 12.5% Tricine-SDS-PAGE followed by staining with silver reagents (inset). The release of 2800 cpm of [14C]Glc in the control was taken as 100% activity.

**Glucosidase I Contains Distinct Domain Structure**—Among the proteases tested, trypsin cleaved the purified glucosidase I into fragments of 69, 55, and 39 kDa; small amounts of 29- and 16-kDa fragments were also observed. The purified enzyme was digested with trypsin for different time intervals, and the fragmentation pattern and the enzyme activity were determined. After 15 min of proteolysis, the enzyme yielded three fragments of 69, 55, and 39 kDa. Extended proteolysis resulted in a marked accumulation of the 39-kDa fragment accompanied by a sequential decrease in the intensities of 69- and 55-kDa fragments (Fig. 4A). The accumulation of a 39-kDa fragment with concomitant decrease in the larger fragments indicates a precursor-product relationship between different fragments. A possibility existed that trypsin digestion of glucosidase I might have generated additional polypeptides that might not be immunoreactive. This was examined by staining the trypsin-generated peptides, after SDS-PAGE, with silver reagents (Fig. 4B). The results showed that the 69-, 55-, and 39-kDa polypeptides are the only major fragments of proteolytic digestion. Whereas the 69-kDa fragment was very faint on silver-stained gel, it was clearly visible on the immunoblot. It was interesting to note that even after 60 min of proteolysis, the activity of glucosidase I was found to be 61% of the control, and at this time of incubation the 39-kDa fragment was the major trypsin-generated peptide. These data also suggested that the 39-kDa fragment may contain the catalytic domain and therefore be enzymatically active. To explore this possibility, the purified enzyme was digested with trypsin under conditions that resulted in the complete disappearance of 85, 69, and 55 kDa bands with a concomitant accumulation of the 39-kDa fragment. The reaction mixture was then incubated with N-(5-carboxypentyl)-1-deoxynojirymycin-Affi-Gel 102, a matrix that specifically binds glucosidase I (11). The enzyme activity bound to the affinity gel and could be eluted quantitatively with 100 mM 1-deoxynojirimycin. The Western blot analysis revealed the presence of a 39 kDa band in the eluate (Fig. 5). These results confirm that the 39-kDa fragment contains the catalytic domain.

**Membrane Orientation of Catalytic Domain**—To address the orientation of the catalytic domain of the enzyme within the membranes, we examined its sensitivity to inhibitors and proteases in intact and detergent-permeabilized microsomal vesicles. Inhibition of glucosidase I with NEM was examined with sealed membrane vesicles in the presence and absence of detergents. There was no significant inhibition of enzyme activity when intact membranes were used. However, the
enzyme activity was inhibited severely by increasing concentrations of NEM when membranes were disrupted with Lubrol PX. This inhibition was abolished completely if an excess of dithiothreitol was included in the reaction milieu. A similar inhibition by NEM also was observed in the presence of saponin (Fig. 6), a detergent that permeabilizes the membranes without disrupting their integrity (31). These results indicate that NEM inhibited the enzyme by reacting with a site on the polypeptide that is protected by the membranes, but is accessible in the presence of the detergent. The inhibition of the enzyme activity in saponin-permeabilized membrane vesicles also suggested that the site affected by NEM may be luminally oriented.

Intact membranes were incubated with increasing concentrations of trypsin on ice in the presence and absence of detergent. The enzyme activity was not affected when the detergent was excluded; however, in its presence, up to 40% inhibition in activity was observed at trypsin concentration as high as 100 μg/ml (Fig. 7). This would be possible if proteolytic products are also enzymatically active. The immunoblotting of these samples revealed that in the absence of detergent, the degradation of the enzyme was minimal and under these conditions, only a single fragment of 82 kDa was generated. On the other hand, in presence of the detergent, the 85-kDa polypeptide was degraded sequentially to a 39-kDa fragment, indicating that the tryptic cleavage at these sites was protected by membranes. These results favor a luminal orientation of a proteolytically resistant catalytic domain.

Several integral membrane enzymes contain luminally oriented catalytic domains and, in some cases, the catalytic domains could be released from saponin-permeabilized membranes by limited proteolysis (33, 34). The sealed and saponin-permeabilized microsomal vesicles were subjected to limited proteolysis with trypsin at 20 °C to release the potential...
catalytic domain of glucosidase I. The digestion had no effect on the total enzyme activity in either intact or saponin-permeabilized membranes (Fig. 8); the activity was found exclusively in the pellet. These results suggest that while saponin allows access to the lumen of ER, the permeabilization does not solubilize the enzyme from the membranes. The digestion in presence of saponin, however, released only 18% of the enzyme activity from the membranes. Under these conditions, i.e. incubation with trypsin at 20 °C in contrast to incubation at 0 °C for the experiment in Fig. 7, trypsin concentrations higher than 50 μg/ml caused disruption of the integrity of membrane vesicles (28, 35) and were found unsuitable for examining the release of higher levels of glucosidase I. Western blot of the samples derived from this experiment revealed that the trypsin digestion of intact membrane vesicles resulted in the conversion of the 85-kDa polypeptide into a polypeptide of approximately 82 kDa that still was associated with the membranes. This confirmed that the cleavage had occurred on the cytoplasmically exposed portion of the enzyme. Digestion of saponin-permeabilized membranes with trypsin resulted in membrane-associated fragments of 69 and 55 kDa and a soluble fragment of 39 kDa, suggesting that the proteolysis occurred on sites facing the lumen of ER (Fig. 8).

The release of enzymatically active 39-kDa fragment from ER membranes could be attributed to the removal of a hydrophobic membrane-anchoring domain. This was confirmed by

![Fig. 7. Inhibition by trypsin treatment. Membranes (4 mg/ml protein) were preincubated in 150 μl of buffer B for 30 min on ice with the indicated amount of trypsin in the absence (A) and presence (B) of Lubrol PX. After the addition of trypsin inhibitor (100 μg/ml), a portion of the sample was incubated with [14C]Glc,Man9GlcNAc2 for the determination of enzyme activity. Release of 8016 and 1050 cpm of [14C]Glc, respectively, was taken as 100%. Samples derived from this experiment were also subjected to Western blot analysis. The latency of mannose-6-phosphatase activity was 97%. The arrow denotes the position of dye front.](image1)

![Fig. 9. Membrane Topography of Glucosidase I](image2)

**FIG. 7. Inhibition by trypsin treatment.** Membranes (4 mg/ml protein) were preincubated in 150 μl of buffer B for 30 min on ice with the indicated amount of trypsin in the absence (A) and presence (B) of Lubrol PX. After the addition of trypsin inhibitor (100 μg/ml), a portion of the sample was incubated with [14C]Glc,Man9GlcNAc2 for the determination of enzyme activity. Release of 8016 and 1050 cpm of [14C]Glc, respectively, was taken as 100%. Samples derived from this experiment were also subjected to Western blot analysis. The latency of mannose-6-phosphatase activity was 97%. The arrow denotes the position of dye front.

**FIG. 8. Release of the catalytic domain of glucosidase I from Saponin-permeabilized ER membranes by limited proteolysis.** Fresh ER membranes (8 mg/ml) in buffer B were treated either with or without 0.5% Saponin in the presence or absence of trypsin (50 μg/ml) at 20 °C for 30 min. After the addition of trypsin inhibitor (100 μg/ml), the reaction mixture was sedimented as described under "Materials and Methods," and enzyme activity in the pellet (P) and supernatant (S) was determined. The values given below the individual lanes in the electrophoretogram represent the percent distribution of the activity in P and S. The release of 5772 cpm for (−saponin−trypsin), 5256 cpm for (−saponin+trypsin), 5538 cpm for (+saponin−trypsin), and 4914 cpm for (+saponin+trypsin) of [14C]Glc, respectively, was taken as 100%. Samples also were analyzed by immunoblotting. The latency of mannose-6-phosphatase activity was 97%.

**FIG. 9. Triton X-114 extracts of ER membranes following trypsin treatment were subjected to phase partitioning ("Materials and Methods").** The aqueous (A) and the detergent (D) layers were concentrated and analyzed by immunoblotting using anti-glucosidase I antibodies and [3H]-labeled anti-rabbit IgG and by determining the enzyme activity. The values given below the figure represent the percent of distribution of the activity in these layers. The release of [14C]Glc, 2886 cpm for undigested sample (−trypsin) and 1788 cpm for trypsin-digested sample (+trypsin), was taken as 100%.

phase separation of proteolysed enzyme with Triton X-114, a detergent whose solutions separate into a detergent phase and an aqueous phase at temperatures above 20 °C (36). Upon phase separation, amphiphatic integral membrane proteins partition into the detergent phase, while hydrophilic proteins partition into the aqueous phase (37). The native glucosidase I partitioned in both the detergent and aqueous phases, suggesting an amphipathic nature. Similarly, 69- and 55-kDa fragments partitioned into both layers. On the other hand, the 39-kDa fragment was highly enriched in the aqueous layer (Fig. 9), favoring its luminal orientation. The native enzyme activity was distributed more or less equally in detergent and aqueous layers. However, after trypsin digestion, the activity in the aqueous layer was almost twice as much as in the detergent phase, consistent with the partitioning of the 39-kDa fragment into the aqueous layer.

The data presented here indicate that glucosidase I is a
transmembrane, endoplasmic reticulum-localized, N-linked glycoprotein with distinct domains within its structure.

**DISCUSSION**

The biosynthesis of glucosidase I occurs in the endoplasmic reticulum. Similar to many other resident proteins of ER, it acquires asparagine-linked, high mannose type oligosaccharide. The results of this study show that the removal of carbohydrate moiety does not seem to affect its catalytic activity significantly. This raises questions regarding the significance of glycosylation of glucosidase I. The requirement for carbohydrate prosthetic groups in the biological activity of glycoproteins has been shown to be variable for different glycoproteins (1). For example, glycosylation of epidermal growth factor receptor has been demonstrated to be essential for its posttranslational activation and ligand-binding capacity (38). On the other hand, the deglycosylation of erythrocyte by glycosidases did not affect its in vitro biological activity in spite of a significant loss of the in vivo activity (39). It is possible that the glycosylation of glucosidase I may be required for its stability, conformation, or retention in the ER.

The major findings of the present study are that glucosidase I is a transmembrane, endoplasmic reticular N-linked glycoprotein; it contains distinct membrane anchoring and luminally oriented catalytic domains; and the enzyme has a cytoplasmically exposed tail. To resolve definitively the question whether glucosidase I is a transmembrane protein, we examined the trypsin accessibility of different regions of this protein in sealed and saponin-permeabilized membranes. The digestion of sealed membranes generated a polypeptide band of approximately 82 kDa from the native enzyme, demonstrating a short cytoplasmic tail. The presence of two bands (85 and 82 kDa) in purified enzyme, therefore, could be attributed to the generation of the 82 kDa band during the solubilization and purification of the enzyme by endogenous proteases even though a mixture of antiproteases was included in all buffers employed. The remaining tryptic cleavage sites appear to be located luminally, because the cleavage at those sites occurred only when membrane vesicles were permeabilized with saponin. The release of enzymatically active 39-kDa fragment from the membranes following trypsinization in the presence of saponin suggests that the catalytic domain is attached to the luminal face of the ER by means of a membrane-anchoring domain. This is consistent with the results of phase partitioning with Triton X-114 which revealed an enrichment of 69- and 55-kDa fragments in the detergent phase and the 39-kDa domain. This is consistent with the results of phase partitioning of the 82 kDa band during the solubilization and purification of the 82 kDa band during the solubilization and purification of the enzyme. It was rather surprising that a removal of more than 50% of the total enzyme mass was required for the change in the phase partitioning behavior. These results raise the possibility that, like glycosyltransferases in the ER (8, 10), glucosidase I also may contain more than one membrane-spanning region. The cDNA sequence of GDP-Man:Man5GlcNAc2-polypeptide (nascent), synthesized on the luminal side of the ER by the oligosaccharyltransferase complex (45). Further, glucosidase II, the enzyme catalyzing the removal of the two glucose α-1,3-linked residues, also appears to be oriented luminally (14). This is in accord with the finding that the newly translated and glycosylated proteins were sequestered within the lumen of the ER (22).

Our current experiments are designed to investigate the detailed structural analysis of oligosaccharide attached to glucosidase I and the role, if any, it plays in the retention of the enzyme in ER.

**Acknowledgments**—We thank Drs. A. Mattoo, A. Mehta, and M. Kang for helpful discussions in preparing this manuscript.

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