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Molecular cloning of cDNAs encoding lamp A, a human lysosomal membrane glycoprotein with apparent M_r ≈120,000

(transmembrane glycoprotein/polylactosamine/hinge region)

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ABSTRACT **Although several lysosomal membrane glycoproteins have been characterized by using specific antibodies, none of the studies so far elucidated the amino acid sequence of a lysosomal membrane glycoprotein. Here we describe cDNA clones encoding for one of the *l*ysosome-*a*ssociated *m*embrane *p*roteins with apparent M_r ≈120,000, lamp A. The amino acid sequence based on the fully coded cDNA shows that as many as 18 potential N-glycosylation sites can be found in the total of 385 amino acid residues. The results obtained by endoglycosidase F digestion support the conclusion that this glycoprotein contains 18 N-glycans. These N-glycosylation sites are clustered in two domains; one contains 10 and the other contains 8 N-glycosylation sites. These domains are separated by a (proline-serine)-rich region that has a distinct homology to the IgA hinge structure. The first N-glycosylated domain is elongated to a potential leader peptide toward the NH_2-terminal end. The second N-glycosylated domain, on the other hand, is connected to a putative transmembrane portion consisting of hydrophobic amino acids. This segment, in turn, is elongated to a short cytoplasmic segment composed of 11 amino acid residues at the COOH-terminal end.**

The lysosomal membrane plays a vital role in the proper function of lysosomes by sequestering numerous acid hydrolases, which are responsible for the degradation of foreign materials and for specialized autolytic phenomena (1). The lysosomal membrane is presumably involved in various important functions of the lysosome, such as its resistance to degradation by lysosomal hydrolases and its ability to interact and fuse specifically with other membrane organelles, including endosomes, phagosomes, and plasma membranes (2). The lysosomal membrane also maintains an acidic intralysosomal environment and at the same time transports low molecular weight products of lysosomal hydrolases (3, 4). Although significant progress has been made in understanding the biosynthesis and targeting of lysosomal hydrolases (5–7), much less is known about the components of the lysosomal membrane.

We have recently characterized a highly sialylated glycoprotein with M_r ≈115,000, leukosialin, which contains a large number of O-linked saccharides (8, 9). When we attempted to isolate glycoproteins in this molecular weight range from chronic myelogenous leukemia cells, we also isolated two highly sialylated glycoproteins that do not bind to anti-leukosialin antibodies coupled to Sepharose. We found that these two glycoproteins belong to lysosomal membrane glycoproteins with M_r ≈110,000, of which some characteristics have been described from several laboratories (10–13). In this paper, we report the complete amino acid sequence for one of the glycoproteins deduced from cDNA analysis and discuss its structure.§

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EXPERIMENTAL PROCEDURE

Isolation of Lysosomal Membrane Glycoproteins. The glycoprotein fraction was isolated by applying the total lysate of chronic myelogenous leukemia cells to wheat germ agglutinin-Sepharose and eluting with 100 mM *N*-acetylglucosamine solution. The eluted material was then subjected to preparative gel electrophoresis, and the material migrating between M_r 105,000 and 130,000 was eluted. These procedures are essentially the same as those for isolating leukosialin (9). Lysosomal glycoproteins were obtained by passing the glycoprotein fraction over a column of the anti-leukosialin antibodies conjugated to Sepharose. The purified glycoproteins that were not bound to this column were used as a starting material for peptide isolation and immunization of rabbits.

Protein Sequence Analysis. The purified glycoproteins were subjected to CNBr fragmentation after *S*-carboxymethylation of the cysteine residues. The resulting peptides were fractionated on a Hypersil ODS (C_{18}) column by using a gradient from 0% to 60% of acetonitrile in 0.1% trifluoroacetic acid. The fractionated peptides were further purified by gel filtration on Sephacryl S-200. Similarly, tryptic peptides were purified by gel filtration on Sephacryl S-200; this was followed by the reverse-phase HPLC. The isolated peptides were sequenced by automated Edman degradation using an Applied Biosystems (Foster City, CA) model 470A sequence; this was followed by identification of phenylthiohydantoin amino acid derivatives by HPLC (14).

Construction of Oligonucleotide Probes and Screening of the cDNA Library. Among several peptides sequenced, two peptides (peptides 1 and 2) were chosen for constructing probes, and oligonucleotides 1 and 2, based on most probable codon (15, 16), were synthesized on an Applied Biosystems automated 380A DNA synthesizer. The oligonucleotides were purified on reverse-phase HPLC and end-labeled with γ-^{32}P (>7000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) by using T4 kinase to a specific activity of $>2 \times 10^9$ cpm/μg. After purification on a NACS column (Bethesda Research Laboratories), the radioactive oligonucleotide was used for colony hybridization screening of a human fetal lung fibroblast cell line (IMR-90) λgt11 cDNA library (17).

Nucleotide Sequencing. Sequencing of cDNA was performed by the phage M13/dideoxynucleotide chain-termination method of Sanger *et al.* (18) by using a kit supplied by Pharmacia and deoxyadenosine 5′-[^{35}S]triphosphate. Following use of universal primers, sequencing was continued

Abbreviations: endo H, endoglycosidase H; endo F, endoglycosidase F.

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§The sequence reported here is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03263).

by use of sequentially constructed 16- to 23-mer oligonucleotides (19).

General methods, including isolation of DNA or RNA, have been described (19, 20).

Immunoprecipitation and Endoglycosidase Digestion. Lysosomal membrane glycoproteins were immunized in rabbits as described (8). Immunoprecipitation of the glycoproteins and endoglycosidase H (endo H) or endoglycosidase F (endo F) digestion followed a previously described procedure (8). The glycoproteins were digested by endo-β-galactosidase as described (21).

RESULTS AND DISCUSSION

Isolation and Pulse–Chase Labeling of Lysosomal Membrane Glycoproteins. During the systematic studies on membrane glycoproteins that contain polylactosaminoglycan, we have isolated highly sialylated glycoproteins with M_r ≈120,000. As shown in Fig. 1*A*, this glycoprotein fraction appeared to be homogeneous by $NaDodSO_4$ gel electrophoresis and also was susceptible to endo-β-galactosidase, suggesting that the glycoprotein carries polylactosaminoglycan (21, 22). The antibodies produced against this glycoprotein fraction were used for immunoprecipitation of metabolically labeled cells. Fig. 1*B* shows that this glycoprotein(s) is synthesized as a precursor of M_r ≈85,000 and this precursor form is converted to a mature glycoprotein of M_r ≈125,000. When the precursor form was digested by endo H or endo F for different periods of incubation, a ladder of bands was revealed (Fig. 1*C*). The results indicate that the glycoprotein contains 16–18 N-linked saccharides. This glycoprotein was detected in all human nucleated cells tested. These results were strikingly similar to those reported on lysosomal membrane glycoproteins (10–13). In parallel, we have obtained evidence that this glycoprotein fraction contains two different glycoproteins by isolating two sets of cDNA clones (see below), which is consistent with the presence of two lysosomal membrane glycoproteins of this molecular weight (11).

To confirm that these glycoproteins are lysosomal membrane glycoproteins, sequential immunoprecipitation was attempted by using our antibodies and monoclonal antibodies. The two monoclonal antibodies (provided by J. T. August, Johns Hopkins University) react with each of the human lysosomal membrane glycoproteins, which are analogous to mouse lamp 1 and lamp 2 (11). The results clearly indicate that the glycoprotein fraction isolated by us consists of two lysosomal glycoproteins, lamp A and lamp B (Fig. 2). Immunocytochemical studies by electron microscope using our antibodies confirmed the localization of glycoproteins in lysosomal membranes (J. Roth and M.F., unpublished data). Furthermore, we have more recently isolated a monoclonal antibody that reacts with lamp B. Lamp A and lamp B could be separated by applying the glycoproteins to an antibody-Sepharose column (S.R.C. and M.F., unpublished data).

Screening of the cDNA Library. Since we were not aware that the glycoprotein fraction isolated was a mixture of two lysosomal membrane glycoproteins, we isolated peptides after CNBr fragmentation of the glycoprotein fraction. Among the obtained peptide sequences, two were used to design large oligonucleotide probes based on the most probable used codons (15, 16). The screening of the human fetal lung fibroblast λgt11 cDNA library provided two sets of different cDNA clones, and each cDNA was hybridized only to those within the same set of cDNAs. In the first attempt, we decided to characterize the first set of cDNA clones, which is based on the peptide 1 sequence (Fig. 3*A*). The glycoprotein encoded by this cDNA is called lamp A. Three different overlapping clones were isolated and sequenced by using the M13/dideoxynucleotide method, and the exact oligonucleotide (corresponding to nucleotides 283–306 of the λ-lamp A in Fig. 3*B*) was used as above to isolate the λ-lamp A-4 clone. The sequence of the insert in this clone is shown

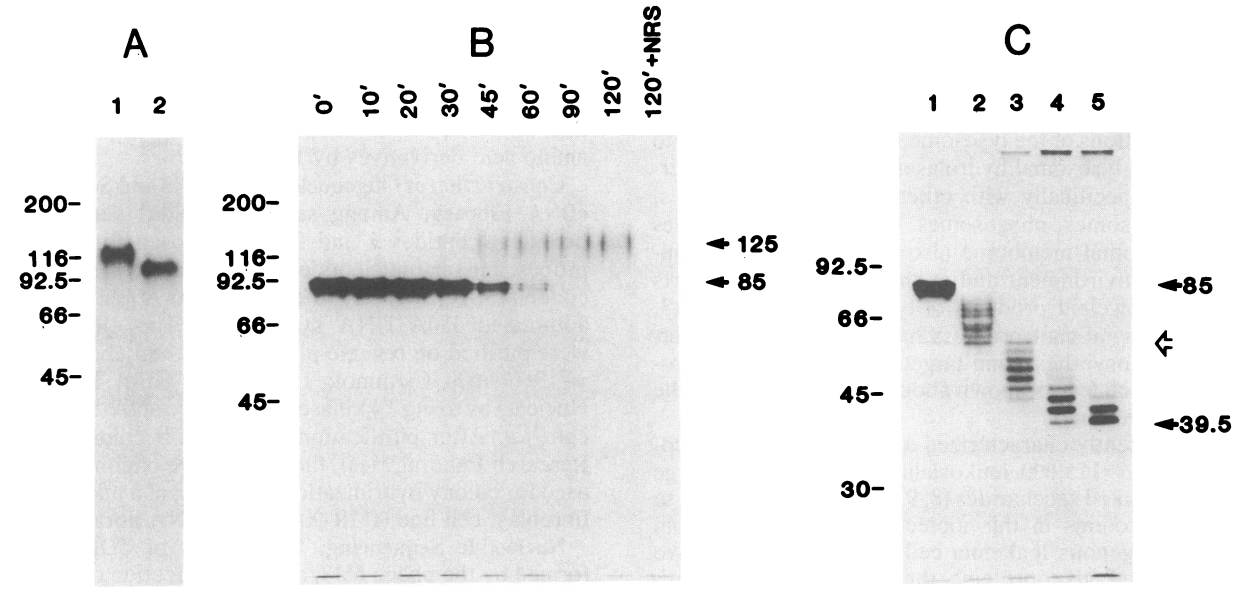

FIG. 1. Characteristics of the lysosomal glycoproteins with M_r ≈120,000. (*A*) Analysis of the purified glycoprotein fraction before (lane 1) and after (lane 2) endo-β-galactosidase treatment. The glycoprotein purified from chronic myelogenous leukemia cells was iodinated with $Na^{125}I$ and subjected to $NaDodSO_4$/polyacrylamide gel (7.5–12.5% gradient) electrophoresis; this was followed by autoradiography. (*B*) Pulse–chase labeling experiment with HL-60 cells. Cells were labeled with [^{35}S]methionine for 10 min and chased with unlabeled methionine for indicated periods of time (8). Lysates were prepared and immunoprecipitated with the specific antibodies. As a control for nonspecific binding, the sample taken after a 120-min chase was immunoprecipitated with preimmune serum [120′ + NRS (normal rabbit serum)]. (*C*) Partial digestions of the glycoprotein precursor forms with endo F. HL-60 cells were labeled with [^{35}S]methionine for 10 min and chased for 10 min with unlabeled methionine. The glycoprotein precursors were immunoprecipitated and either were untreated (lane 1) or followed by treatment with endo F for 5 min (lane 2), 20 min (lane 3), 45 min (lane 4), and 24 hr (lane 5). Treatment with endo H gave similar results. The open arrow shows an unrelated contaminating protein from HL-60 cells. In a separate experiment, two more upper bands were noticed after a 5-min digestion. Analyses in *B* and *C* were made by $NaDodSO_4$/polyacrylamide gel electrophoresis (9% acrylamide) and fluorography. Molecular weights are given as $M_r \times 10^{-3}$.

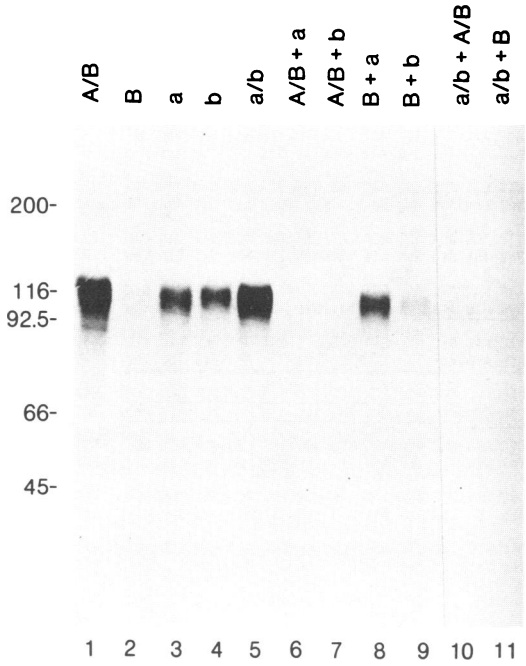

FIG. 2. Immunoprecipitation of lysosomal membrane glycoproteins by antibodies specific to lamp A and lamp B. [^{3}H]Glucosamine-labeled K562 cells were lysed and immunoprecipitated with anti-lamp (A plus B) (lane 1) or anti-lamp B (lane 2) (prepared by us) or with anti-lamp A (lane 3), anti-lamp B (lane 4), or anti-lamp A plus anti-lamp B antibodies (lane 5) (provided by J. T. August). Sequential immunoprecipitation (lanes 8 and 9) provides the evidence that our anti-lamp B antibody actually immunoprecipitates lamp B defined by J. T. August. Lanes 6, 7, 10, and 11 are also the result of sequential immunoprecipitation. A/B or a/b denotes a mixture of two antibodies and "+" denotes sequential immunoprecipitation. The immunoprecipitation by anti-lamp B antibody was inhibited by the presence of the purified glycoproteins shown in Fig. 1*A* (lane 2). Molecular weights are given as $M_r \times 10^{-3}$.

in Fig. 3*B*. The same sequence was obtained from two independent cDNA clones isolated from the IMR-90 library.

Predicted Primary Sequence and Structure of the Lysosomal Membrane Glycoprotein. The sequence of the fully coded cDNA for lamp A had an open reading frame of 1155 nucleotides. The nucleotide sequence in the vicinity of the methionine at position 25 (CCGCCATGG) corresponds to the consensus initiation site for translation (23). The codon usage in this reading frame was similar to the reported one from human coding sequences (15) (Fig. 3*B*). Starting from the aforementioned methionine, the translation product is a 385-amino acid polypeptide. Sequences at positions 85–104, 132–166, and 283–287 were also found in the amino acid sequences obtained from CNBr fragments and tryptic fragments of the initial protein preparation. The cDNA insert hybridized to a single species of mRNA from IMR-90 and HL-60 cells with an apparent size of 2.2 kilobases (data not shown).

The open reading frame peptide has a hydrophobic (Fig. 3*B*) segment near the COOH terminus. The length of 24 amino acids of this hydrophobic region is capable of spanning the membrane (24). It is delineated with basic amino acid residues toward the COOH-terminal end, which is a consensus sequence for "halt-transfer" domains (24). The cytoplasmic segment is short, consisting of only 11 amino acids. Comparable sizes of cytoplasmic segments have been described in other membrane glycoproteins (25). In the NH_2-terminal region right after the initiation site, we found a considerably hydrophobic domain of 16 amino acids, which appear to be a potential leader peptide. The amino acid sequence around position 17 conforms with the leader peptide proteolytic cleavage site "(−3, −1)" rule (26). The presence of three arginine residues close to the initiation methionine (26) also supports the idea that the peptide segment of 16 amino acids is probably a leader peptide. However, it is not clear at this point whether this leader peptide is actually cleaved.

The amino acid sequence contains 18 consensus sequences for N-linked glycan attachment sites (Fig. 3) that are organized into two different clusters separated by a (proline-serine)-rich region (the solid line box in Fig. 3). Each cluster contains four cysteine residues, which may be linked by disulfide bonds. If all of the possible glycosylation sites are utilized, the concentration of carbohydrates is especially high in the peptide stretch of the first 100 amino acids. Four of the 18 putative glycosylation sites are within the sequenced CNBr fragments. All of these four sites at 89, 98, 133, and 149 gave a blocked signal in peptide sequencing, indicating that these glycosylation sites are utilized. In addition, the partial digestion by endo F confirms that this glycoprotein contains 18 N-glycosylation sites (Fig. 1*C*).

Although two independent cDNA clones from the IMR-90 library provided the same nucleotide sequence, the sequence around the NH_2-terminal region needs further clarification. This is because lamp A isolated from chronic myelogenous leukemia cells showed an NH_2-terminal sequence different from the sequence deduced from the cDNA (Fig. 3*C*). It appears that there is an additional sequence composed of 12 residues starting from alanine in lamp A isolated from chronic myelogenous leukemia cells. It is likely that lamp A in IMR-90 cells is coded by mRNA spliced differently from that of chronic myelogenous leukemia cells.

Homologous Region to the IgA α_1-Chain Hinge Region. A characteristic 23-amino acid-long stretch with high contents of proline (11/23) and serine (6/23) begins starting at residue 164. The amino acid sequence of the CNBr fragment could be extended until residue 170. It confirmed that proline was not hydroxylated at least in the beginning of this domain. However, one of four serines gave a very low signal, suggesting a posttranslational modification, probably O-glycosylation at those points (see below).

A data base search for possible homologous proteins to lamp A revealed the homologous sequence in this (serine-proline)-rich region (Fig. 4). It is intriguing that this region has a distinct homology with the IgA α_1-chain hinge region (27). It has been shown that this hinge region is glycosylated by O-linked saccharides (28). It appears that two human tumor viruses, Epstein–Barr virus (29) and human T-lymphotropic virus type II (30), show similar hinge structures (Fig. 4).

Although lysosomal membrane glycoproteins were characterized to some extent (10–13), there was no report on the amino acid sequence of a lysosomal membrane glycoprotein and its unique structure. Our results indicate that the lamp A consists of two heavily N-glycosylated domains separated by a hinge region. It is possible, but unlikely, that this large amount of carbohydrate is serving as a marker for targeting the glycoprotein to lysosomes. In fact, the glycoprotein is not phosphorylated, in agreement with a previous report (12). This bulky carbohydrate probably protects the lysosomal glycoprotein from degradation by various proteases in the luminar side of lysosomes (13). The isolated glycoprotein contains a significant amount of polylactosaminoglycan with sialic acid, suggesting that the glycoprotein is sorted out from the trans-Golgi cisternae.

It is interesting to compare present results with those obtained on mannose-6-phosphate receptors, which also reside in prelysosome compartments. The mannose-6-phosphate receptors bind to lysosomal enzymes in the Golgi apparatus, and the receptor–ligand complex is transported to a prelysosomal compartment where lysosomal enzymes are dissociated from the receptors. The receptors then recycle to

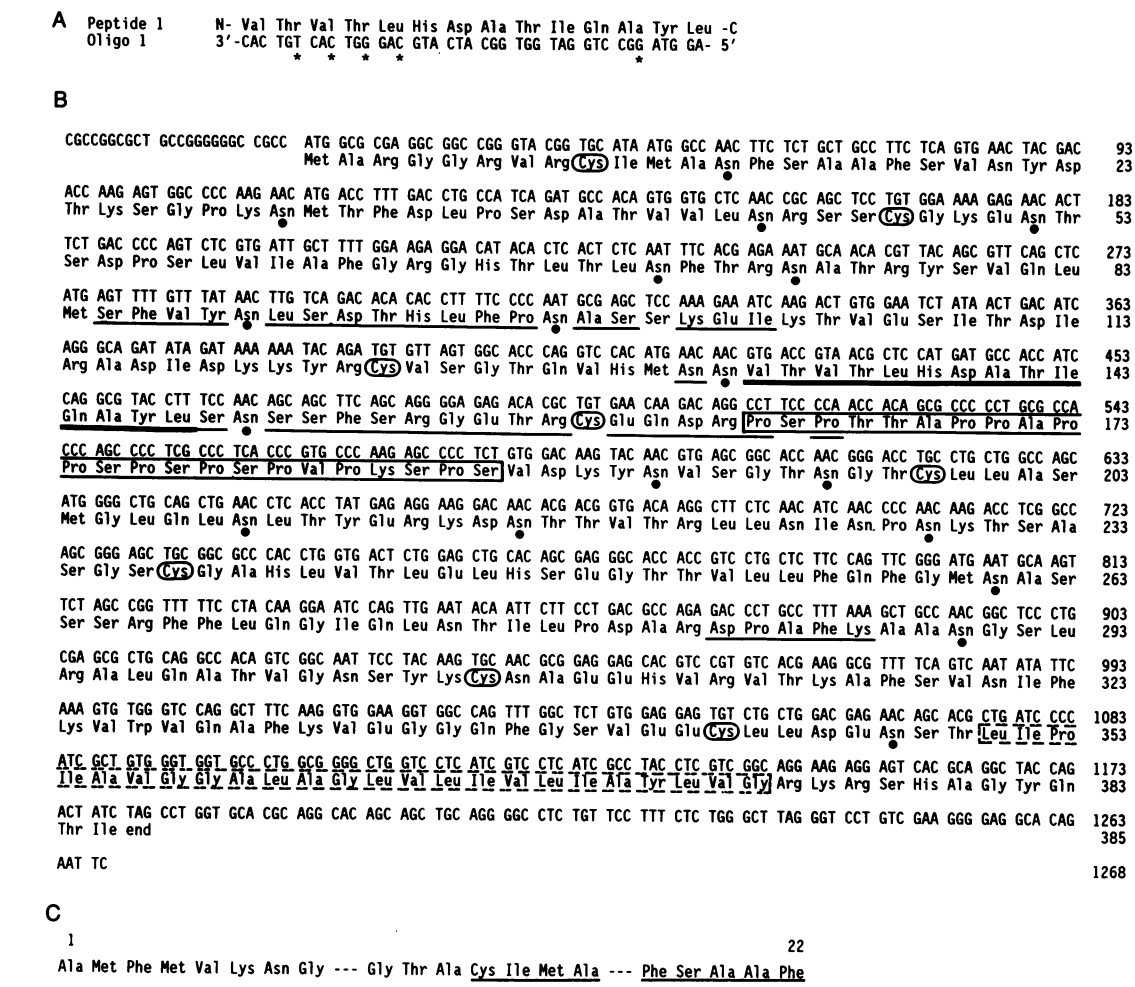

FIG. 3. Oligonucleotide probe used for screening the λgt11 library (*A*) and the nucleotide sequence and deduced amino acid sequence of the lamp A cDNA clones (*B*). (*A*) Peptides were isolated by CNBr fragmentation of the protein and fractionated by HPLC. The amino acid sequences of the peptides were used to construct the oligonucleotide probe based on the most probable codons. The positions indicated by stars differ from the actual sequence shown in *B*. (*B*) The sequence of the insert for the total coding region, λ-lamp A-4, and deduced amino acid sequence are shown. The same nucleotide sequence was obtained from two independent clones. The amino acid sequence obtained from peptide analysis is underlined. The positions that were shown as blank during the amino acid sequencing are not underlined. Two regions of the sequence corresponding to the (serine-proline)-rich hinge region (solid line) and the putative transmembrane portion (dashed line) are indicated by boxes. Cysteine residues are circled and potential N-glycosylation sites are indicated by dots. (*C*) NH_2-terminal sequence of lamp A isolated from chronic myelogenous leukemia cells. Residues 9 and 17 gave no signal during amino acid sequencing. Note that residues 13–22 (underlined) are identical to residues 9–18 in the sequence deduced from cDNA.

the Golgi apparatus and repeat the process (31, 32). On the other hand, lysosomal membrane glycoproteins may function in the fusion process with various other organelles. In fact, immunoelectron micrographic examination detects the gly-

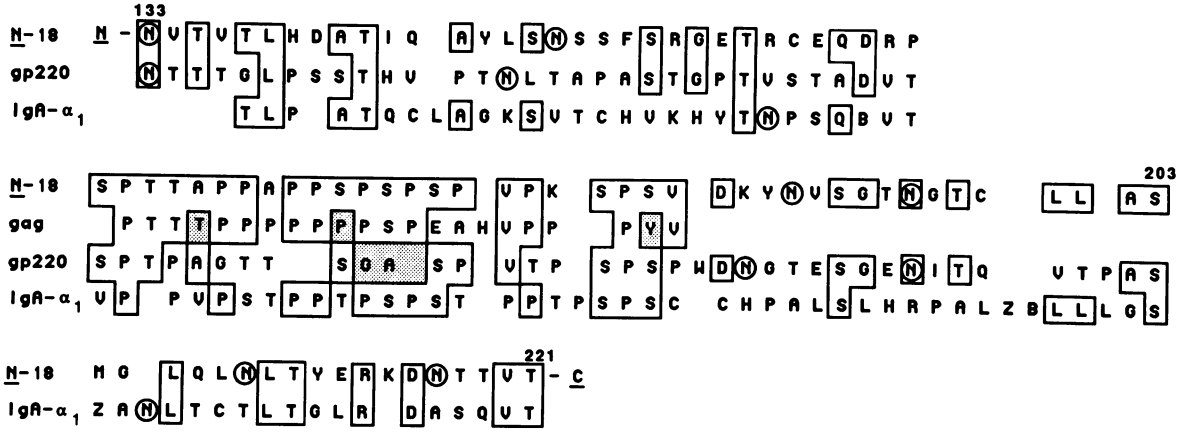

FIG. 4. Comparison of the lamp A peptide sequence (N-18) with homologous sequences from human T-lymphotropic virus type II gag protein (gag), Epstein–Barr virus membrane glycoprotein gp220 (gp220), and IgA α_1 chain (IgA-α_1). The homologies with existing protein primary structures were tested by using the Microgenie (Beckman) data base. Identical amino acids are shown as boxed and N-linked glycan attachment sites are circled. The amino acid sequence in IgA α_1 chain is in its hinge region (27).

coprotein in plasma membranes and multivesicular bodies as well (J. Roth and M.F., unpublished results). In support of this contention, we did not detect any homology in the amino acid sequences between the lysosomal membrane glycoprotein and the mannose-6-phosphate receptors (33–35). The availability of cDNA coding for the full length of lamp A will allow us to understand the molecular mechanisms of targeting lysosomal membrane glycoproteins to lysosomes and the possible role of carbohydrates in this glycoprotein.

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