Biosynthesis, Glycosylation, Movement through the Golgi System, and Transport to Lysosomes by an N-Linked Carbohydrate-independent Mechanism of Three Lysosomal Integral Membrane Proteins

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The biosynthesis, glycosylation, movement through the Golgi system, transport to lysosomes, and turnover of three lysosomal integral membrane proteins (LIMPs) have been studied in normal rat kidney cells using specific anti-LIMP monoclonal antibodies. Immunoelectron microscopy studies revealed the presence of LIMPs in secondary lysosomes, Golgi cisterna, and coated and uncoated vesicles located in the trans-Golgi area. Pulse-chase experiments recorded LIMP precursors of 27 (LIMP I), 72 (LIMP II), and 86 kDa (LIMP III) and mature LIMPs of 35-50 (LIMP I), 74 (LIMP II), and 90-100 kDa (LIMP III). Time course studies on the acquisition of endoglycosidase H resistance by LIMPs indicated that all three LIMPs moved from the site of their synthesis in the endoplasmic reticulum to the medial Golgi within 30-60 min after their synthesis. All three LIMPs were fully glycosylated before leaving the Golgi system, the process during which LIMP I was retained in the trans side of the organelle. LIMP I reached the lysosomes with a half-time of 2 h and LIMPs II and III with half-times of 1 h after their synthesis by a mechanism that was independent of N-linked carbohydrates. LIMPs free of N-linked carbohydrates displayed much shorter half-lives than fully glycosylated LIMPs, suggesting an important role of the sugars in protecting LIMPs against proteolytic degradation. Double immunofluorescence microscopy experiments showed that LIMP I, LIMP II, and LIMP III are localized in the same lysosomes.

The biogenesis of lysosomes is a complex process that requires the synthesis and processing of soluble and membrane components, their sorting from other newly synthesized cellular elements, and finally their integration into new or pre-existing lysosomes. In recent years great progress has been made in the knowledge of the biosynthesis, processing, and mechanism of sorting of the main soluble lysosomal components, the acidic hydrolases (reviewed in Refs. 1 and 2). These proteins are co-translationally inserted into the lumen of the endoplasmic reticulum and then transported to the Golgi system together with other secretory and membrane proteins. In this organelle they acquire mannose 6-phosphate in the N-linked high-mannose oligosaccharide chains (3-5). Recognition of the mannose 6-phosphate residues by specific membrane receptors located in the cisterna of the Golgi system results in sorting and delivery of the enzymes to lysosomes (6, 7). The enzymes are proteolytically processed during their transport and upon their arrival in lysosomes (8, 9; revised in Ref. 2).

Much less is known about the biosynthesis, processing, mechanism of sorting, and delivery of integral membrane proteins (LIMPs) to lysosomes. Although recently some LIMPs have been characterized using polyclonal and monoclonal antibodies (10-13), the information about those processes is still very limited. To address some of these questions we have obtained monoclonal antibodies against LIMPs with which we have studied the molecular characteristics, biosynthesis, processing, movement from endoplasmic reticulum to lysosomes, and degradation of three of these proteins. Pulse-chase experiments show that after their synthesis LIMPs acquire N-linked high-mannose and complex carbohydrates as shown by their sensitivity to endo H and endo F digestion. Study of the rates of acquisition of endo H resistance indicate that all three LIMPs move from the cis to the trans side of the Golgi system with similar velocities. LIMPs are transported from Golgi to lysosomes with different rates by a mechanism that is independent of N-linked carbohydrates. Furthermore, LIMPs display different half-lives and the carbohydrates acquired in the Golgi system protect LIMPs against proteolytic degradation in the lysosomes. The implications of these results in the biogenesis and degradation of lysosomes are discussed.

EXPERIMENTAL PROCEDURES

RESULTS

Development of Monoclonal Anti-LIMP Antibodies and Cellular Localization of the Antigens—Screening of hybridoma clones for producers of anti-LIMPs antibodies was performed by indirect immunofluorescence microscopy using NRK cells.

1 The abbreviations used are: LIMP, lysosomal integral membrane protein; BSA, bovine serum albumin; endo H, endo-β-N-acetylglucosaminidase H; endo F, endo-β-N-acetylglucosaminidase F; GIMP, Golgi integral membrane protein; dGLIMP, lysosomal integral membrane protein without N-linked carbohydrates; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; NRK, normal rat kidney.

2 Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 2, 3, and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2233, cite the authors, and include a check or money order for $6.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Fig. 1. Cellular localization of LIMPs. The cellular localization of LIMPs in NRK cells (a–d) and primary somatotrophs from rat pituitary cells (e–f) was studied by immunoelectron microscopy, using the monoclonal anti-LIMP I antibody, 14E12, as first antibody and peroxidase-conjugated goat anti-mouse F(ab')2, as second antibody. Cells studied with the monoclonal anti-LIMP II antibody, 29G10, or the monoclonal anti-LIMP III antibody, 38C7, exhibited the same pattern of organelle staining as with antibody 14E12. a, note the exclusive localization of LIMP I in lysosomes. Endoplasmic reticulum, mitochondria, nuclear membrane, and plasma membrane are not stained by the anti-LIMP I antibody (bar = 1 μm). b, detail of two secondary lysosomes of different size (bar = 0.5 μm). c, localization of LIMP I in the Golgi system: note the light and heavy, respectively, staining of the central parts and rims (arrowheads) of the cisterna (bar = 0.25 μm). d, localization of LIMP I in Golgi cisterna (arrowhead) and coated (large arrow) and uncoated vesicles (small arrow) budding from the cisterna (bar = 0.25 μm). e, staining of a rat somatotroph with anti-LIMP I antibody. Note the large accumulation of small vesicles (i.e. primary lysosomes) loaded with LIMP I in the trans side of the Golgi system (bar = 0.25 μm). f, LIMP I-containing primary lysosomes approaching (small arrows) and fusing (arrowheads) with the secretory granules of a rat somatotroph (bar = 0.25 μm).

The selected clones secreted antibodies that exclusively stained cytoplasmic vesicles predominantly clustered around the nucleus (see Fig. 9). Three of these clones, 14E12, 29G10, and 38C7, produced antibodies that specifically recognized three different LIMPs (see below). When studied by immunoelectron microscopy in NRK cells, the antibodies were found to stain mainly large secondary lysosomes (Fig. 1, a and b). Occasional staining of Golgi cisterna (Fig. 1, c and d), coated vesicles probably budding from the trans-cisterna, and uncoated vesicles located in the trans-Golgi area were also observed (Fig. 1, c and d). The small size vesicles without coat display characteristics of primary lysosomes as shown in
studies with pituitary cells. These cells in the absence of hypothalamic releasing factors displayed an extensive process of lysosome-dependent c areas involving secretory granules (34). As a result the cells exhibited large numbers of primary lysosomes. These organelles were positively stained with anti-LIMP antibodies and consisted of small vesicles distributed throughout the cytoplasm that were frequently found in the vicinity of or fusing with secretory granules (Fig. 1, e and f). The fusion event probably starts the degradation of the granules and the formation of secondary lysosomes.

**Molecular Characterization of LIMPs and Localization of LIMP Epitopes—See Miniprint Section.**

**Rate of LIMPs Transport through the Golgi System—**The acquisition of sialylated N-linked complex carbohydrates by LIMPs clearly indicated that these proteins traversed the entire Golgi system in a cis to trans direction. To determine the rate of this movement, the time course of acquisition of endo H resistance by the mature LIMPs was studied (Fig. 4). We observed that after half an hour of chase most of LIMP III displayed the same resistance to endo H exhibited by its mature form after 1.5 h of chase. A substantial part of LIMP I and LIMP II also displayed endo H resistance by this time. By 1 h of chase all the LIMP II and III exhibited the partial resistance to endo H characteristic of their mature forms whereas after 1 h and 1.5 h of chase small amounts of LIMP I still displayed a sensitivity to endo H that was not observed in the mature form. It is noteworthy that all the endo H-resistant forms of LIMPs were sensitive to neuraminidase after 1 h of chase (not shown). All these results indicated that LIMPs moved from the cis to the trans side of the Golgi with similar but not identical rates. A careful study of the electrophoretic mobility of LIMPs revealed that whereas LIMP II and LIMP III did not experience changes in their molecular weights after acquiring the endo H resistance, LIMP I still exhibited a slight increase in it (Fig. 4). It is important to note that endo F digests of LIMP I immunoprecipitated either immediately after acquiring endo H resistance or after complete processing displayed similar molecular weights (Fig. 2, panel A, compare lanes 3 with 9). Furthermore, metabolic labeling of LIMP I with the sialic acid precursor [6-3H]N-acetylmannosamine resulted in the exclusive labeling of forms of the protein with apparent molecular mass higher than 45 kDa (data not shown). These results suggest that slow sialylation could be responsible for the retarded processing of LIMP I.

**Transport of LIMPs from the Golgi System to Lysosomes—**To study the transport of LIMPs from the Golgi system to lysosomes the proteins were metabolically labeled with [35S]methionine, chased for different periods of time, and their distribution between the low density Golgi elements and high density lysosomes analyzed after separating the organelles by Percoll gradients (35). Fig. 5 (see Miniprint Section) shows the separation of Golgi elements and lysosomes by Percoll gradients.

The transport of LIMPs from the Golgi system to lysosomes is described in Fig. 6. Operationally the transport rate is defined as the time required for a newly synthesized LIMP to move from the fractions containing the Golgi elements to the fractions containing lysosomes. The half-time of transport is calculated as the time required for 50% of the newly synthesized protein to consummate the movement. It can be observed that half an hour after their synthesis, the precursors and mature forms of LIMPs were located in the fractions containing the Golgi elements. However, a small amount of mature LIMP II was already detected in lysosomes by that time. As the time of chase was increased the amount of mature LIMPs associated with lysosomes became larger and a parallel decrease of their presence in the fractions containing the Golgi elements was recorded. These results indicated that LIMPs were effectively transported from the Golgi system to lysosomes. By 2 h of chase the transport of LIMPs II and III was virtually completed as indicated by their most exclusive localization in the lysosomal fractions. Interestingly, only 40 and 70% of LIMP I was found associated with lysosomes after 2 and 4 h of chase. The prolonged presence of LIMP I in the fractions containing Golgi and plasma membrane elements could result from retention of the protein in the Golgi system or transport of the protein to the plasma membrane. These two possibilities were examined in different experiments. In a first experiment, cells metabolically labeled with [35S]methionine for 15 min and chased with cold methionine for 0, 1, 2, 3, and 4 h were incubated with anti-LIMP I antibody for 1 h at 4 °C and the incorporation of newly synthesized LIMP I into the plasma membrane studied by immunoprecipitation (36). No 35S-labeled LIMP I was immunoprecipitated by the
were immunoprecipitated with the monoclonal antibodies anti-LIMP
thionine for 0.5, 1, 2, and gradients after different times as described in the legend to Fig. 5. Fractions of the Percoll gradients
visualized by fluorography, and quantitated by laser scanner densi-
solved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis,
thesized LIMP labeled for 15 min with \[^{35}\text{S}\]methionine were chased with cold methionine for 0.5, 1, 2, and 4 h. After the chase the cells were processed as described in the legend to Fig. 5. Fractions of the Percoll gradients were immunoprecipitated with the monoclonal antibodies anti-LIMP I, 14E12, anti-LIMP II, 29G10, anti-LIMP III, 39C7, and anti-GIMP 130, 15C8. The immunoprecipitated \[^{35}\text{S}\]labeled antigens were re-
solved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, visualized by fluorography, and quantitated by laser scanner densi-
tometry. The experiment shown in the Figure is one of two experi-
ments that produced similar results.

In a second experiment, cells labeled as before were incubated for 30 min at 4 °C in the absence and presence of proteinase K and the levels of \[^{35}\text{S}\]labeled LIMP I in the fractions of the Percoll gradient containing Golgi elements and plasma membrane compared. No significant differences in the levels of LIMP I were found between the fractions obtained from untreated and proteinase K-treated cells. These results suggested that the prolonged presence of newly syn-
thesized LIMP I in the light fractions of the Percoll gradient was the result of its retention in the Golgi system before being delivered to lysosomes. This agrees with the observation that glycosylation of LIMP I continued after acquiring resistance to endo H, a process which is likely to result in retention of the protein in the trans side of the Golgi system. The selectivity of the transport of LIMPs to lysosomes was examined by comparing their distribution in Golgi elements and lyso-

somes with that of a 130-kDa Golgi cisterna integral membrane protein (GIMP), characterized in our laboratory.3 As can be seen in Fig. 6, there was no detectable amount of GIMP in lysosomes after 2 h of chase. This result indicated the existence of specific mechanisms of sorting and transport that selected the membrane proteins delivered to lysosomes.

Transport of LIMPs from the Golgi System to Lysosomes in Tunicamycin-treated Cells—Study of the effect of the N-
glycosylation inhibitor tunicamycin on the transport of LIMPs from the Golgi system to lysosomes was of considerable interest for two reasons. The conceivable involvement of N-linked oligosaccharides in this process, and the possibility that the transport of LIMPs to lysosomes could be coupled to the mannose 6-phosphate-dependent transport of lysosomal enzymes. These studies were performed with the same pro-
tocol used to study the transport of LIMPs in normal cells with the difference that 5 μg/ml tunicamycin and 10 μg/ml leupeptin were continuously present in the cell culture medium. Henceforth the N-linked oligosaccharide free forms of LIMPs will be referred to as dGLIMPs. Study of the transport of dGLIMP I revealed that 80% of the protein was already in lysosomes 2 h after its synthesis (Fig. 7). A similar rate of transport was recorded for dGLIMP III. The rapid transport of dGLIMP I was in contrast with the considerably slower rate of movement of the fully glycosylated LIMP I (see above). This difference, the evidence that LIMP I is retained in the Golgi system and the similar rates of transport displayed by dGLIMP I and dGLIMP III supported the suggestion that glycosylation of LIMP I and not its movement from Golgi to lysosomes was the rate-limiting step in its transport to lysosomes. It is relevant that in contrast with dGLIMP I, apparently stable during the time required to study its transport, dGLIMP III was immediately degraded in lysosomes. Degra-
dation resulted in production of two 33- and 23-kDa peptides that were immunoprecipitated by the anti-LIMP III antibody from the lysosomal fractions. The transport of dGLIMP II to lysosomes could not be determined with certainty. Although a significant decrease in the amount of dGLIMP II associated with Golgi elements was detected in chase experiments, a parallel increase was not observed in lysosomes. This result probably reflected the rapid degradation of dGLIMP II in lysosomes. Nevertheless, we cannot rule out the possibility that dGLIMP II is not transported to lysosomes and it is degraded somewhere else. It is noteworthy that the Golgi protein, 130-kDa GIMP, was localized in Golgi elements in

\[\text{I\, Time of chase} \quad \text{LIMP I} \quad \text{LIMP II} \quad \text{LIMP III} \]

\[\begin{array}{c|c|c}
0.5 h & p & p \\
1 h & p & p \\
2 h & p & p \\
4 h & p & p
\end{array}\]

\[\text{Bottom} \quad \text{Top} \quad \text{Bottom} \quad \text{Top} \quad \text{Bottom} \quad \text{Top}\]

\[\begin{array}{c|c|c}
0.5 h & p & p \\
1 h & p & p \\
2 h & p & p \\
4 h & p & p
\end{array}\]

\[\text{Bottom} \quad \text{Top} \quad \text{Bottom} \quad \text{Top} \quad \text{Bottom} \quad \text{Top}\]

\[\text{Golgi gp130} \quad \text{2 h} \quad \text{2 h} \quad \text{2 h} \quad \text{2 h} \quad \text{2 h} \]

\[\text{FIG. 6. Distribution of pulse \[^{35}\text{S}\]-labeled LIMPs in Percoll gradients after different times of chase. NRK cells metabolically labeled for 15 min with \[^{35}\text{S}\]methionine were chased with cold methionine for 0.5, 1, 2, and 4 h. After the chase the cells were processed as described in the legend to Fig. 5. Fractions of the Percoll gradient containing Golgi elements were immunoprecipitated with the monoclonal antibodies anti-LIMP I, 14E12, anti-LIMP II, 29G10, anti-LIMP III, 39C7, and anti-GIMP 130, 15C8. The immunoprecipitated \[^{35}\text{S}\]-labeled antigens were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, visualized by fluorography, and quantitated by laser scanner densitometry. The experiment shown in the Figure is one of two experiments that produced similar results.}\]

\[\text{FIG. 7. Distribution in Percoll gradients of pulse \[^{35}\text{S}\]-labeled LIMPs chased for different times in the presence of tunicamycin. NRK cells preincubated for 2 h in the presence of 5 μg/ml tunicamycin and 10 μg/ml leupeptin, were pulse-labeled with \[^{35}\text{S}\]methionine for 15 min and chased for 0.5, 1, and 2 h, both pulse and chase in the presence of tunicamycin and leupeptin. After the chase the cells were processed as described in the legend to Fig. 5 and the amounts of \[^{35}\text{S}\]-labeled LIMPs in the fractions of the gradients determined as described in the legend to Fig. 6. The experiment shown in the Figure is one of two experiments that produced comparable results.}\]
cells treated with tunicamycin (not shown) indicating that the absence of carbohydrate did not affect its normal distribution and was not transported to lysosomes for degradation. Therefore, the results clearly showed that LIMPs displayed different half-lives, the shortest of them corresponding to LIMP I with 8 h and the longest one to LIMP II with 20 h, with LIMP III exhibiting a half-life of 10 h (Fig. 8). Furthermore, the half-lives of dGLIMP I, 4 h, dGLIMP II and dGLIMP III, 1 h, were considerably shorter than those of the fully glycosylated LIMPs (Fig. 8). These results were consistent with the hypothesis that N-linked carbohydrates play a role in protecting LIMPs against proteolytic degradation. This role could be of physiological importance given the specific localization of LIMPs in lysosomes and the high concentration of proteolytic enzymes in these organelles.

Co-distribution of LIMPs in Lysosomes—Having found that LIMPs were transported to lysosomes at different rates and displayed different half-lives, we considered the possibility that these differences could reflect their localization in different lysosomes. To study this possibility we analyzed the distribution of the three LIMPs in lysosomes by double immunofluorescence microscopy. As shown in Fig. 9 these experiments revealed a complete coincidence in the cellular distribution of LIMPs, indicating that LIMPs I, II, and III were located in the same lysosomes.

![Fig. 8. Half-lives of fully glycosylated and N-linked carbohydrate-free LIMPs.](image)

![Fig. 9. Co-localization of LIMPs in the same lysosomes.](image)

NRK cells were studied by double immunofluorescence using the monoclonal anti-LIMP antibodies 14E12 (LIMP I) and 29G10 (LIMP II). In a first step, the cells were incubated with anti-LIMP I antibody, this was tagged with rhodamine-conjugated goat anti-mouse antibody and both cross-linked to LIMP I using formaldehyde. In a second step, the cells were incubated with anti-LIMP II antibody and then with fluorescein-conjugated goat anti-mouse antibody. Complete correlation between the distribution of the two anti-LIMP antibodies was interpreted as evidence that LIMP I and LIMP II were localized in the same lysosomes as the tagged rhodamine-conjugated antibody was cross-linked to the LIMP I site and could not have reacted with any LIMP II located in different lysosomes. A, rhodamine channel; B, fluorescein channel. Note the identical cellular distribution of LIMP I and LIMP II in vesicles clustered around the nucleus that were identified by electron microscopy as lysosomes. Control experiments in which the first or second antibody steps were omitted revealed no rhodamine fluorescence in the fluorescein channel and vice versa. Identical results were obtained in studies of the co-distribution of LIMP I and II with III.

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**Processing Transport Turnover of Lysosomal Membrane Proteins**

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DISCUSSION

We have developed and used monoclonal antibodies against integral membrane proteins from lysosomes to study the cellular distribution, molecular characteristics, processing, movement from the endoplasmic reticulum to lysosomes, and turnover of these proteins. The aim of these studies is to increase our knowledge of the biogenesis and biodegradation of lysosomes.

The three LIMPs here characterized contain N-linked carbohydrates. LIMP I apparently contains exclusively complex-type carbohydrates, whereas LIMPs II and III contain complex as well as high-mannose oligosaccharides. Stialylated-complex carbohydrates have been detected in all three LIMPs. We have not found any evidence of the presence of O-linked carbohydrate chains in LIMPs as shown by their insensitivity to O-glycosidase. However, it cannot be discarded that the small increase in the apparent molecular weight of LIMP III during pulse-chase experiments performed in the presence of tunicamycin could correspond to the acquisition of O-linked carbohydrates by the protein.

Several lysosomal membrane proteins have been recently characterized using monoclonal and polyclonal antibodies; two proteins, LAMP I and LAMP II, have been identified in mouse lysosomes (11), one, Igpl20, in rat (10) and one, gp95-105, in chicken lysosomes (12). Three of these proteins, LAMP I, Igpl20, and gp95-105 and the rat LIMP III here described, display precursors and mature forms with similar apparent molecular weights and isoelectric points. Although the reported cellular localizations of these proteins are not identical (LAMP I (37) and gp95-105 (12) are both expressed on the plasma membrane whereas LIMP III is exclusively found in lysosomes) it cannot be excluded that they could be the same protein. It is also not clear whether any of these proteins is kindred to the 100-kDa protein from lysosomes studied with a polyclonal antibody and immunologically related to the K\textsuperscript{+}-H\textsuperscript{+}-ATPase pump from gastric mucose (13). No monoclonal antibody has been previously obtained against LIMP II. This protein could be related to other similar molecular weight that has not been characterized and was immunoprecipitated with several other membrane proteins by a polyclonal antibody prepared against lysosomal membranes (10). With respect to LIMP I there is no report of a lysosomal membrane protein with its characteristics.

The movement of LIMPs from the site of their synthesis in the endoplasmic reticulum to and through the Golgi system has been examined by studying the conversion of N-linked high-mannose carbohydrates to complex carbohydrates. Only proteins reaching the trans-Golgi have their N-linked oligosaccharide chains converted to sialylated complex carbohydrates, due to the location of β-galactosyltransferase (38) and sialyltransferase (32, 33) in this part of the Golgi system. The finding that LIMPs I, II, and III, as well as other LIMPs previously described (10, 12, 39) acquire N-linked complex carbohydrates containing sialic acid, strongly indicates that they cross the entire Golgi in a cis to trans direction. It is important to note that the movement of LIMPs from the endoplasmic reticulum through the cis and to the medial-Golgi, where endo H resistance is acquired (40), is performed with similar speeds (30–60 min). Furthermore, whereas the speeds are comparable to that of other membrane (41) and soluble proteins, including some lysosomal hydrolases (42), that traverse the Golgi system, they are different from those of other proteins performing the same movement (43, 44). It is noteworthy that whereas the processing of LIMP II and LIMP III is terminated immediately after acquiring endo H resistance, that of LIMP I slowly continued. The localization of sialyltransferase in the trans-Golgi (32, 33) and the incorporation of sialic acid to the sugars of LIMP I displaying high apparent molecular weights, suggest that the slow processing of LIMP I is probably due to the late acquisition of sialic acid residues. There is considerable controversy about the site in the Golgi system from which different lysosomal components are delivered to lysosomes. Lysosomal hydrolases have been proposed to be released from cis-Golgi (45), trans-Golgi (46, 47), and even from an independent organelle called GERL (48). The facts that LIMPs cross the entire Golgi system and that LIMP II and LIMP III are transported to lysosomes immediately after reaching the trans-Golgi indicates that most likely LIMPs are delivered to lysosomes from the trans side of the Golgi system.

Study of the transport of LIMPs from the site of their synthesis in the endoplasmic reticulum to a dense lysosomal compartment reveals that two of these proteins, LIMP II and LIMP III are transported with a half-time of 1 h, whereas LIMP I is transported with a half-time of 2 h. The movement of LIMP II and III from the endoplasmic reticulum to the trans-Golgi is a characteristic of the Golgi system in 30–90 min and their presence in lysosomes 30 min after their synthesis, indicate that these two LIMPs are transported to lysosomes immediately after their arrival to the trans-Golgi. In contrast, the delayed presence of LIMP I in lysosomes 2 h after its synthesis and 90 min after its arrival to the trans-Golgi compartment indicates that it is probably retained in the trans-Golgi (see above).

It is important to note that the rates of transport of LIMPs to lysosomes are within the range of those reported for soluble lysosomal enzymes in different systems (42, 49, reviewed in Ref. 2). The different rates with which some lysosomal hydrolases are transported from the endoplasmic reticulum to lysosomes (50) could also be due to different processing in the Golgi system. However, there are important differences between the mechanisms of delivery of LIMPs and soluble lysosomal enzymes from the Golgi system to lysosomes. LIMPs are not phosphorylated and at least two dGLIMPs, dGIMP I and dGIMP III, are transported to a dense lysosomal compartment in the presence of tunicamycin, a drug that completely blocks the transport of lysosomal hydrolases from the Golgi system to lysosomes (51). These results indicate that transport of LIMPs from the Golgi system to lysosomes is not mediated by the mannose 6-phosphate residues that, contained in N-linked high-mannose oligosaccharide chains, instrument the transport of lysosomal hydrolases. They also indicate that the transport of LIMPs is not coupled to that of soluble lysosomal hydrolases and that it is independent from N-linked carbohydrates. This independence is in contrast with the role played by N-linked carbohydrates in the movement of some glycoproteins to the plasma membrane (52). The absence of O-linked carbohydrates from the LIMPs here studied also seems to discard a role of these sugars in the transport. It is likely that the putative signals for sorting and delivery of LIMPs to lysosomes reside in the amino acid sequence, tertiary structure, or in the same type of post-translational modification of these proteins. In contrast, there is a marked effect of carbohydrate on the stability of LIMPs as demonstrated by the observation that dGLIMPs synthesized in the presence of tunicamycin display much shorter half-lives than the fully glycosylated mature LIMPs. In fact the differences probably are larger than the ones measured by us here as the half-lives of LIMPs were measured in the absence of protease inhibitors whereas those of dGLIMPs

* I. Sandoval, unpublished results.
were measured in the presence of leupeptin. Clearly, dGLIMP III is rapidly degraded by proteolysis upon its arrival to lysosomes and although the site of the accelerated degradation of dGLIMP I and dGLIMP II has not been determined, it cannot be discarded that it is in lysosomes. These results are consistent with the general observation that glycoproteins are degraded faster after deglycosylation (reviewed in Ref. 53). They also point to a fundamental role of the carbohydrate moieties in protecting LIMPs from degradation by lysosomal hydrolases as has been hypothesized previously by others (10). This may also explain why in normal cells only fully glyco-sylated LIMPs are delivered to lysosomes. The immunoelec-
tron microscopy studies of the cellular distribution of LIMPs in NRK cells agree with the biochemical evidence indicating that LIMPs cross the entire Golgi system before being delivered to lysosomes from the trans side of this organelle. In these studies LIMPs are found to be present in several Golgi cisternae, coated vesicles probably budding from the trans most cisterna and in uncoated vesicles located in the trans-Golgi area. It is not clear whether LIMPs are transported from Golgi to dense lysosomes, probably secondary lysosomes, in the same or different vesicles. Our data cannot distinguish between these two possibilities. Double immunoelectron microscopy studies of these vesicles, using anti-LIMP antibodies labeled with different probes could be helpful to solve this problem.

It is important that primary cultured pituitary cells dis-
tain large numbers of LIMP-loaded vesicles similar to the uncoated vesicles found in the trans-Golgi area of NRK cells. They also point to a fundamental role of the carbohydrate moieties in protecting LIMPs from degradation by lysosomal hydrolases as has been hypothesized previously by others (10). This may also explain why in normal cells only fully glyco-
SUPPLEMENTAL MATERIAL TO

BIODEGRADATION, GLYCOSYLATION, MOVEMENT THROUGH THE GOLGI SYSTEM AND TRANSPORT TO LYSOSOMAL CHAMBERS: MECHANISM OF THREE LYSOSONAL INTEGRAL MEMBRANE PROTEINS (LIMPS)

BY

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EXPERIMENTAL PROCEDURES

Cell culture - HK (normal rat kidney) and pituitary cells were grown in GIBCO's modified Eagle's medium (MEM) with F-12/H (1:1) fetal bovine serum (FBS) and glucose free (0.1 g/l) with 1% non-essential antibiotics.

Cell counting - Cells were seeded at 10,000 cells/cm² in 8 wells of a 6-well plate and the medium was replaced every 2 days. After 3 days, the medium was removed and the cells were washed twice with PBS. The cells were then treated for 30 s with 0.1% Trypan blue in PBS and counted in a haemocytometer.

RESULTS

Regulation of LIMP processing - Two additional observations were made in the present study. Firstly, the treatment of cells with 100 ng/ml interferon-gamma (IFN-γ) led to an increase in the expression of LIMP-1 and LIMP-3. Secondly, the treatment of cells with 100 ng/ml TNF-α also led to an increase in the expression of LIMP-1 and LIMP-3.

CONCLUSIONS

The results of the present study indicate that the processing of LIMPs is regulated by several factors, including cytokines. The precise mechanisms by which these factors regulate LIMP processing remain to be elucidated. Further studies are needed to clarify the role of cytokines in the regulation of LIMP processing and to determine the downstream signaling pathways involved.

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Processing Transport Turnover of Lysosomal Membrane Proteins

**Fig. 2.** Characterization of the Precursors and Maturant Forms of LIMP. (A) untreated and tunicamycin-treated (5 μg/ml) NRK cells were pulsed-labeled for 15 min with 35S-methionine and labeled-LIMP immunoprecipitated immediately after or following 1 hour or 2 hours of chase with 1 μM cold methionine, using anti-LIMP I (4422) or anti-LIMP II (2020) or anti-LIMP III (3012) polyclonal antibodies (see Experimental Procedures). When treated with the immunoprecipitates were treated with endo H (1 μg/ml) (lanes 1) or neuraminidase and a mixture of endo F and O-glycosidase (lanes 5). Immunoprecipitates obtained from cells incubated with tunicamycin were either not treated (lanes 1 or treated with neuraminidase and then O-glycosidase (lanes 10). Characterization of the glycoproteins obtained from the immunoprecipitates obtained from NRK cells treated with tunicamycin and chased for 1 hour (lanes 1) or 2 hours (lanes 2) and their partial conversion to 6-N-acetylglucosamine by endo F and endo H (lanes 6 and 7). Notice the absence of O-glycosylated carbohydrate in LIMP I (compare lanes 7 and 8) and LIMP III (compare lanes 11 and 12) and the partial conversion of the 6-N-acetylglucosamine by endo F and endo H (lanes 6 and 7). Notice the absence of O-glycosylated carbohydrate in LIMP I (compare lanes 7 and 8) and LIMP III (compare lanes 11 and 12) and the partial conversion of the 6-N-acetylglucosamine by endo F and endo H (lanes 6 and 7). Notice the absence of O-glycosylated carbohydrate in LIMP I (compare lanes 7 and 8) and LIMP III (compare lanes 11 and 12) and the partial conversion of the 6-N-acetylglucosamine by endo F and endo H (lanes 6 and 7). 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