The EMBO Journal vol.8 no.12 pp.3633 – 3640, 1989

Lysosomal acid phosphatase is transported to lysosomes via the cell surface*

Martin Braun, Abdul Waheed and Kurt von Figura

Institut für Biochemie II, Gosslerstrasse 12d, D-3400 Göttingen, FRG

Communicated by K.von Figura

*This study is dedicated to Hans-Dieter Söling on the occasion of his 60th birthday.

Lysosomal acid phosphatase (LAP) is transported as a transmembrane protein to dense lysosomes. The pathway of LAP to lysosomes includes the passage through the plasma membrane. LAP is transported from the trans-Golgi to the cell surface with a half-time of <10 min. Cell surface LAP is rapidly internalized. Most of the internalized LAP is transported back to the cell surface. On average, each LAP molecule cycles >15 times between the cell surface and the endosomes before it is transferred to dense lysosomes. At equilibrium ~4 times more LAP precursor is present in endosomes than at the cell surface. Exposing cells to reduced temperature or weak bases such as NH_4Cl, chloroquine and primaquine decreases the steady-state concentration of LAP at the cell surface. The recycling pathway is operative at ≥20°C and does not include passage of the Golgi/trans-Golgi network. LAP is transferred with a half-time of 5–6 h from the plasma membrane/endosome pool to dense lysosomes, from where it does not recycle to the endosome/plasma membrane pool at a measurable rate.
Key words: endosomes/lysosomal membrane glycoproteins/lysosomes/plasma membrane/protein targeting

Introduction

It has recently become apparent that several lysosomal membrane glycoproteins have some structural features in common which define them as a distinct class of membrane glycoproteins. They are characterized by a high number of sialylated N-linked oligosaccharides attached to their luminal domain, a single membrane-spanning domain and a relatively short carboxy-terminal tail of 10 or 11 residues protruding into the cytoplasm (Chen *et al.*, 1988; Fambrough *et al.*, 1988; Fukuda *et al.*, 1988; Howe *et al.*, 1988; Viitala *et al.*, 1988). Lysosomal acid phosphatase (LAP) shares several features with this class of lysosomal membrane glycoproteins. It is synthesized as a transmembrane protein with seven or eight N-linked oligosaccharides, a single transmembrane domain and a cytoplasmic tail of 18 amino acids (Pohlmann *et al.*, 1988; Waheed *et al.*, 1988). Its transport to lysosomes is independent of oligosaccharide processing and mannose 6-phosphate receptors, and is not affected by weak bases (Gottschalk *et al.*, 1989a). Newly synthesized LAP is transported with a half-time of 6–7 h to dense lysosomes. There LAP is proteolytically processed to a soluble protein of the lysosomal matrix. The proteolytic processing occurs with a half-time of 6–7 h and involves at least two sequential cleavages. The first is catalysed by a thiol proteinase at the outside of the lysosomal membrane that removes the bulk of the cytoplasmic tail, and the second by an aspartyl proteinase within the luminal domain close to where the polypeptide chain exits from the membrane (S.Gottschalk *et al.*, 1989b).

In the present study we have examined the transport of LAP from the trans-Golgi to dense lysosomes. We show that LAP is transported to the cell surface from where it is rapidly endocytosed. LAP at the cell surface and in endocytic structures is in equilibrium through rapid recycling. The steady-state concentration of LAP at the cell surface can be decreased by exposing cells to reduced temperature, chloroquine, NH_4Cl or primaquine. On average, LAP persists for 5–6 h in the plasma membrane/endosome pool before it is transferred to dense lysosomes.

Results

Cell surface associated LAP

After cell surface iodination of BHK cells overexpressing human LAP, a diffusely appearing polypeptide with an apparent size of 67 kd was precipitated from the cell extract with LAP antiserum (Figure 1A). This polypeptide was

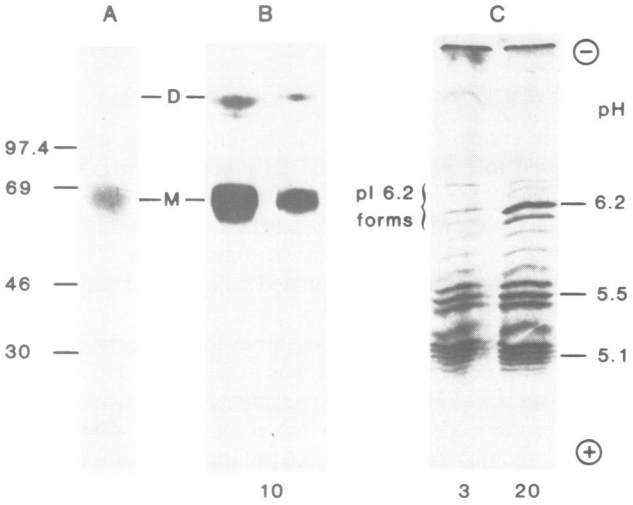

Fig. 1. Presence of LAP at the cell surface. (**A**) Monomeric (M) and dimeric (D) forms of LAP immunoprecipitated from cells that had been iodinated at 4°C. Numbers at the left margin refer to the positions of mol. wt standards (kd). (**B**) LAP immunoprecipitated from the surface (**right**) and the remaining cell extract (**left**) of cells that had been labelled and chased for 2 h each. LAP accessible to antibodies accounted for 10% of total LAP. (**C**) Cells that had been labelled and chased for 2 h each were incubated for 60 min at 4°C in the absence (**left**) or presence (**right**) of neuraminidase. LAP was immunoprecipitated from the Triton X-114 extract of the cells and subjected to IEF. The pH gradient and the electrodes are indicated. The p*I* 6.2 forms account for 3% of membrane-associated LAP in the control and for 20% in the cells treated with neuraminidase, indicating that LAP accessible to neuraminidase accounts for 17% of membrane-associated LAP.

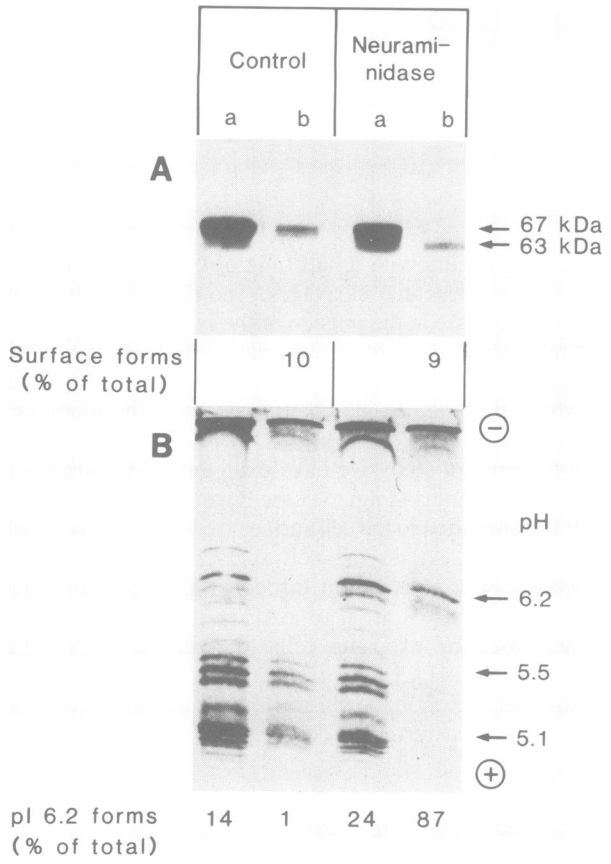

Fig. 2. Sensitivity of cell surface associated LAP to neuraminidase. Cells labelled and chased for 2 h each were incubated at 4°C without or with neuraminidase. LAP immunoprecipitated from the surface (**lane b**) and the remaining cell extract (**lane a**) was separated by SDS–PAGE (**A**) or IEF (**B**). In (A) the sialylated (67 kd) and desialylated (63 kd) monomeric forms of LAP and the percentages of LAP accessible to antibodies at 4°C (surface LAP) are indicated; and in (B) the percentages of p*I* 6.2 forms of LAP are shown.

absent in controls reacted with pre-immune serum and is considered to represent the 67 kd precursor of LAP.

To quantify the fraction of LAP present at the cell surface, BHK cells were metabolically labelled, chilled to 4°C and incubated for 1 h at 4°C with neuraminidase. The LAP precursor was immunoprecipitated from the Triton X-114 extract of cells and separated by isoelectric focusing (IEF). As the LAP precursor was sensitive to treatment of cells at 4°C with neuraminidase it was assumed to derive from the cell surface. Preliminary experiments had shown that IEF separates the LAP precursor into at least 12 distinct forms with p*I*s ranging from 4.9 to 5.6 and that incubation of the Triton X-114 extract of cells at 37°C with neuraminidase converts the acidic forms of LAP into three major (basic) forms with p*I*s around 6.2. This shift in p*I* is based on the removal of sialic acid residues which are present in the N-linked oligosaccharides attached to the luminal (ectoplasmic) domain of LAP. Cells were labelled for 2 h and then chased for 2 h to ensure that most of the labelled LAP precursor has passed the compartment of sialyltransferase. Twenty per cent of the LAP precursor from cells that were incubated with neuraminidase at 4°C was recovered after IEF at pH 6.2 as compared to 3% in controls incubated with buffer (Figure 1C). This indicates that 17% of the LAP precursor is accessible at 4°C to neuraminidase and hence located at the cell surface. In seven independent determinations this value varied from 13 to 21% (mean 18%). The small amount of p*I* 6.2 forms in the controls is attributed to LAP precursor that has not passed the compartment of sialyltransferase. This is based on the observation that non-sialylated LAP precursors in the endoplasmic reticulum have the same p*I* as desialylated LAP precursors (not shown).

Cells were incubated at 4°C with neuraminidase, washed and the solubilized cells were incubated for 4 h at 4 or 37°C to test whether residual neuraminidase, which had not been removed by the washing procedure, desialylates intracellular LAP after solubilization of the cells. The IEF pattern of LAP

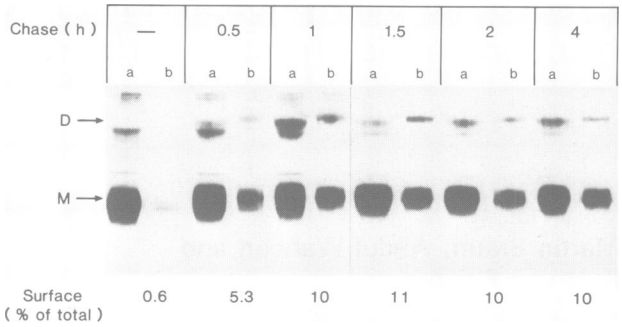

Fig. 3. Kinetics of LAP transport to the cell surface. After labelling for 30 min and a chase of up to 4 h, LAP accessible to antibodies at the cell surface (**b**) and from the remaining cell extract (**a**) were immunoprecipitated. The fraction of LAP recovered in (b) is given below the lanes as a percentage of total LAP. It may be noted that initially monomeric (M) and dimeric (D) LAP forms are ~6 and ~12 kd smaller in size than after prolonged chase. This increase in size is due to processing of the oligosaccharides (Waheed *et al.*, 1988).

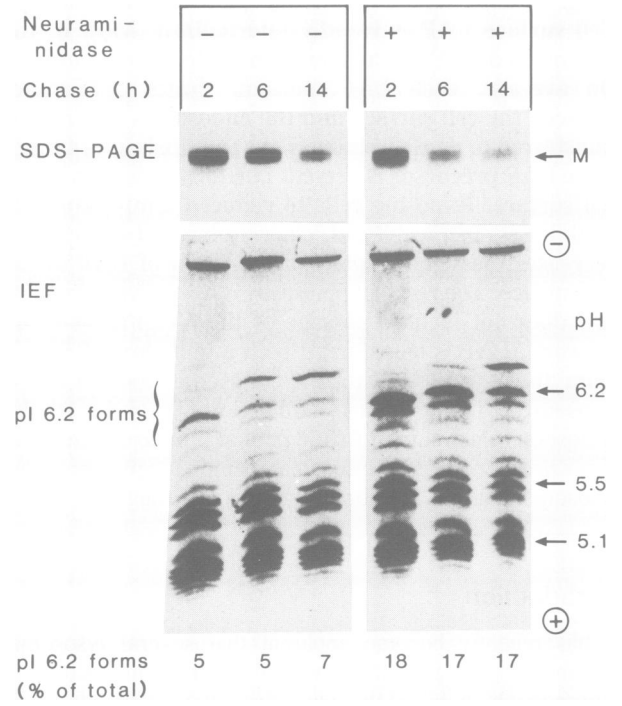

Fig. 4. Persistence of LAP at the cell surface. Cells were labelled for 2 h and chased for 2–14 h prior to incubation at 4°C without (**left**) or with (**right**) neuraminidase. The LAP was immunoprecipitated from the Triton X-114 extract of the cells and separated by SDS–PAGE (**A**) or IEF (**B**). For symbols and numbers below lanes see Figures 1 and 2.

isolated directly after the solubilization or after the incubation for 4 h at 4 or 37°C was identical (not shown). This indicates that desialylation of intracellular LAP after solubilization of the cells is negligible.

Furthermore, we determined the fraction of LAP accessible to antibodies at 4°C. In cells labelled and chased for 2 h each, 8–11% of LAP precursor was accessible to antibodies (Figure 1B). If these LAP precursors are located at the cell surface, they should be accessible to neuraminidase at 4°C. Therefore, cells were incubated at 4°C with neuraminidase prior to incubation with LAP antibodies at 4°C. Neuraminidase treatment decreased the size of antibody-accessible LAP (9–10% of total) by ~4 kd (Figure 2A) and shifted its p*I* to 6.2 (Figure 2B). These results show that the antibody procedure is specific for cell surface LAP. However, antibodies bind only ~60% of the LAP precursor that is accessible to neuraminidase. This became apparent in mutants, in which all LAP is located at the cell surface (C.Peters, unpublished) and explains why antibodies precipitate ~10% of the LAP precursor, while neuraminidase desialylates ~18%.

Recycling of LAP precursor

The half-time of the transport of newly synthesized LAP to the cell surface was determined with the antibody approach, since the neuraminidase/IEF assay is not applicable to cells in which most of the labelled LAP precursors are located proximal to the trans-Golgi and are yet not sialylated. In cells labelled for 30 min the amount of cell surface associated LAP reached a plateau within 1.5 h of chase (Figure 3). Thirty minutes were required for half-maximal association of LAP with the cell surface. Since the mean life time of LAP at the beginning of the chase was 15 min, the half-time for transport of LAP to the cell surface is 45 min. We have observed earlier that it takes 37.5 min to transport half of the newly synthesized LAP to the trans-Golgi (Waheed *et al.*, 1988). Transport of LAP from the trans-Golgi to the cell surface requires therefore <10 min. The fraction of cell surface LAP (determined with the neuraminidase/IEF assay) remained constant during a chase for up to 14 h, accounting for 10–13% of total LAP precursor (Figure 4, lower panel). It may be noted that during the 14 h chase, 60% of the labelled LAP precursor was transported to lysosomes and processed to the soluble 52 kd form of LAP (the disappearance of the LAP precursor during the chase is visible in the upper panel of Figure 4. The 52 kd product is recovered in the water phase after Triton X-114 extraction).

The association of a constant fraction of the LAP precursor with the cell surface suggested that precursors at the cell surface and in intracellular membranes are in equilibrium. To demonstrate recycling of LAP, cells were treated three times with neuraminidase at 4°C. After the first and second treatment the cells were incubated for 15 min at 37°C to allow membrane flow. After the first treatment with neuraminidase, 19% of the LAP precursor was desialylated, after the second treatment 42% and after the third treatment 48% (Figure 5). This clearly indicates that during the 37°C intervals sialylated LAP precursor was transferred to the cell surface replacing a comparable amount of cell surface associated LAP that was internalized. When cells that had been incubated at 4°C with neuraminidase were recultured for 15–60 min at 37°C, half of the desialylated LAP forms

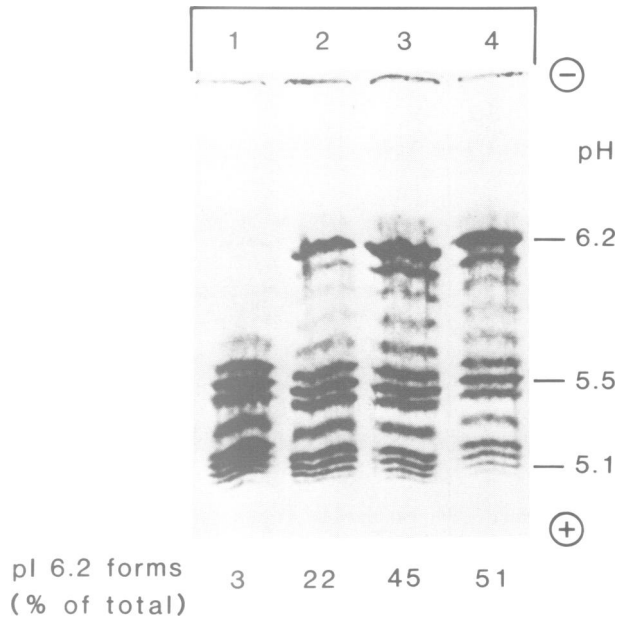

Fig. 5. Recycling of LAP. Cells were labelled and chased for 2 h each and then incubated at 4°C without (**lane 1**) or with (**lane 2**) neuraminidase. Part of the cells treated with neuraminidase were recultured at 37°C for 15 min and subjected to a second treatment at 4°C with neuraminidase (**lane 3**). Cells shown in **lane 4** were subjected to an additional cycle of reculturing at 37°C and neuraminidase treatment at 4°C. LAP was immunoprecipitated from the Triton X-114 extract of the cells and separated by IEF. For symbols and numbers below lanes see Figures 1 and 2.

at the cell surface were replaced within ≤7 min by sialylated forms (Figure 6).

The experiments shown in Figures 5 and 6 underestimate the rate of recycling due to long incubation periods (≥15 min) at 37°C. When the 37°C intervals were shortened to 2.5 min, 47% of LAP was desialylated after three cycles of neuraminidase treatment at 4°C (see Figure 8). This indicates that each LAP precursor is recycled at least once every 20 min. This value may still underestimate the kinetics of recycling. If cells are incubated at 37°C with neuraminidase <3 min are required to desialylate 50% of the LAP (Figure 7). Although at 37°C some desialylation may also occur in endocytic structures, namely upon prolonged incubation due to pinocytosis of neuraminidase, the initial kinetics of desialylation are determined by the accessibility of LAP at the cell surface and the activity of neuraminidase. It is therefore likely that the cycle time of LAP is <20 min and close to 6 min.

The effect of temperature on the recycling was examined by two approaches. When cells treated with neuraminidase at 4°C were recultured for 15 min at 37, 20, 15 or 4°C, sialylated LAP forms, which could be desialylated by a second treatment with neuraminidase at 4°C, were observed only in cells recultured at 20 and 37°C. In the other type of experiment the cells were incubated for 1 h with neuraminidase at various temperatures. When cells were incubated with neuraminidase at 37, 25 or 20°C, ≥70% of the LAP precursor was desialylated, while only 15–20% of the LAP precursor was desialylated at 15, 10 and 4°C. These experiments (not shown) clearly indicate that the bulk of LAP precursor resides in intracellular compartments, from where it can recycle to the cell surface at ≥20°C.

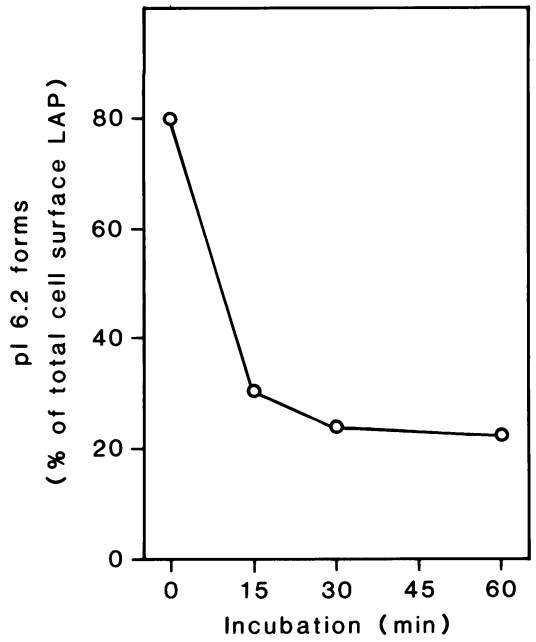

Fig. 6. Replacement of desialylated cell surface LAP by sialylated LAP from internal membranes. Cells labelled and chased for 2 h each were treated at 4°C with neuraminidase. The fraction of LAP accessible to antibodies at 4°C was isolated after the treatment with neuraminidase and after reculturing the cells at 37°C for 15–60 min. The percentage of p*I* 6.2 forms was determined by IEF.

Even upon prolonged incubation of cells at 37°C with neuraminidase, ~10% of the LAP precursor was not desialylated (Figure 7). Fractionation by Percoll density centrifugation of a postnuclear supernatant of cells that had been incubated for 30 min at 37°C with neuraminidase revealed that the sialylated LAP precursors were concentrated in dense lysosomes (not shown). This suggested that the LAP precursors in dense lysosomes only slowly equilibrate—if at all—with the desialylated LAP precursor of the endosome/plasma membrane pool. To examine directly whether desialylated LAP precursors in endosomes equilibrate with sialylated LAP precursors in dense lysosomes, cells were treated three times with neuraminidase at 4°C. After the first and second neuraminidase treatment the cells were kept for 2.5 min at 37°C to allow membrane flow in the absence of neuraminidase. This treatment resulted in desialylation of 47% of the LAP precursor. Percoll density centrifugation showed that the desialylated LAP precursor was present in light membranes, which contain, among others, the markers of endosomes and plasma membrane. In cells kept for 30 min at 37°C after the third treatment with neuraminidase, the desialylated LAP precursors were still concentrated in the light membranes (Figure 8, lower panel). The traces of desialylated forms recovered in dense lysosomes after the chase for 30 min at 37°C were within the range expected from unidirectional transfer of LAP precursor from endosomes to dense lysosomes. A comparison of the precursor and mature LAP forms in the postnuclear supernatant and the Percoll gradient fractions (Figure 8, upper panel) indicates also that a net transfer of LAP precursor from light membranes to dense lysosomes and processing to mature forms during the 30 min chase is hardly detectable. These results suggest that the exchange between LAP precursors in dense lysosomes and endosomes

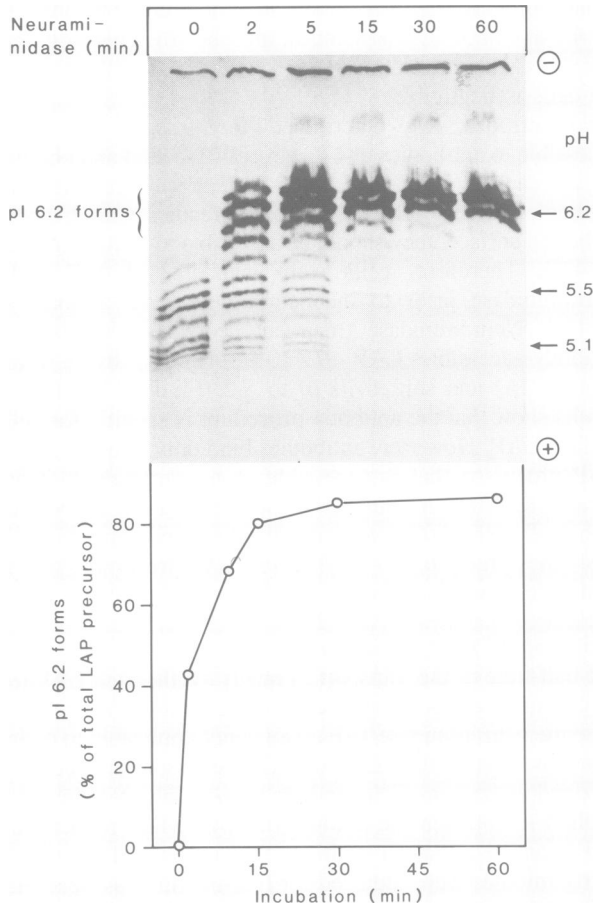

Fig. 7. Desialylation of LAP at 37°C. Cells were labelled and chased for 2 h each and incubated for up to 1 h at 37°C with neuraminidase prior to immunoprecipitation of membrane-associated LAP and separation by IEF (**upper part**). In the **lower part** the percentage of desialylated LAP is plotted versus time.

—if it occurs at all—is much slower than that between endosomes and the plasma membrane.

Recycling does not involve passage of the Golgi

In the following two experiments we examined whether LAP passes the Golgi and/or the TGR during recycling. In the first experiment, cells were labelled and chased for 2 h each in the presence of swainsonine, an inhibitor of Golgi-localized mannosidase II (Tulsiani *et al.*, 1982; Novikoff *et al.*, 1983). Inhibition of Golgi mannosidase II prevents the formation of multiantennary sialylated oligosaccharides and allows only for the formation of sialylated hybrid oligosaccharides. This explains why p*I* 5.1 LAP forms are not synthesized in the presence of swainsonine (Figure 9, lane 1). If recycling includes passage of the Golgi, mannosidase II could initiate further processing of the oligosaccharides in cells chased in the absence of swainsonine. When cells that had been labelled in the presence of swainsonine were chased for 6 h in the absence or presence of swainsonine, the IEF pattern of LAP remained constant (Figure 9, lanes 2 and 3). The failure to detect p*I* 5.1 forms after the chase in the absence of swainsonine suggests that recycling of LAP does not involve passage of the Golgi.

In the second experiment cells that had been incubated at 4°C with neuraminidase were recultured at 37°C for 6 and

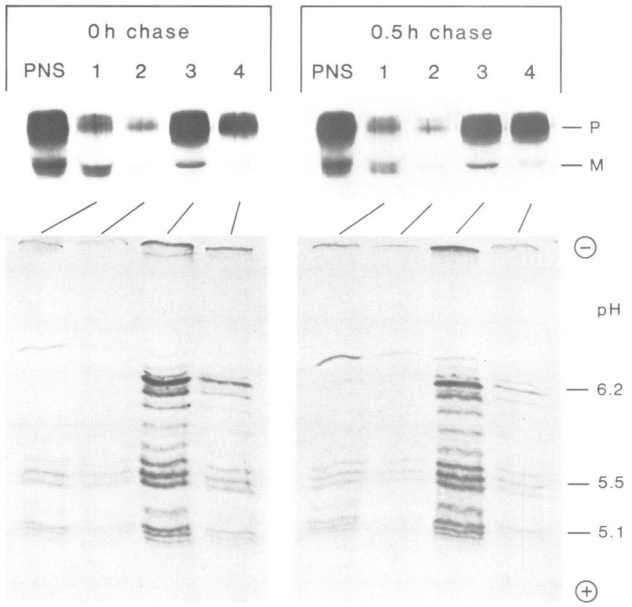

Fig. 8. LAP does not recycle from dense lysosomes to light membranes. Cells labelled and chased for 2 h each were treated three times at 4°C with neuraminidase. After the first and second treatments with neuraminidase the cells were incubated for 2.5 min at 37°C. Cells were harvested after the third neuraminidase treatment (**left**) or after an incubation for 30 min at 37°C (**right**). A postnuclear supernatant (PNS) was prepared and separated into four fractions by Percoll density centrifugation. Fraction 1 contains dense lysosomes, fraction 3 the bulk of light membranes including Golgi, plasma membrane and endosomes. LAP was immunoprecipitated from aliquots of the PNS and the gradient fractions and separated by SDS−PAGE (**upper part**). The **lower part** shows the IEF pattern of LAP immunoprecipitated from the Triton X-114 extract of the gradient fractions. For symbols see Figures 1 and 2.

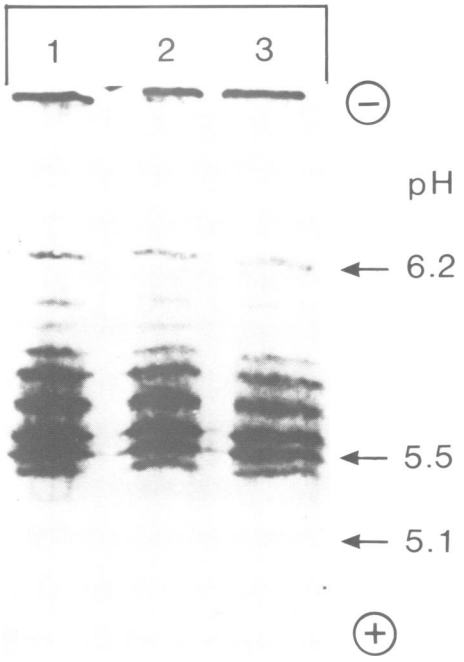

Fig. 9. LAP does not recycle to the site of Golgi mannosidase II. Cells were labelled and chased for 2 h in the presence of swainsonine (**lane 1**). Part of the cells were then chased at 37°C for 6 h in the presence (**lane 2**) or absence (**lane 3**) of swainsonine. The LAP was immunoprecipitated from the Triton X-114 extract and separated by IEF.

12 h. If recycling of LAP would include passage of the trans-Golgi/trans-Golgi network, where sialyltransferase is concentrated (Roth *et al.*, 1985), LAP may become resialylated during the reculturing at 37°C. Resialylation was not detectable (Figure 10), suggesting that recycling does not involve passage of the trans-Golgi/trans-Golgi network.

Weak bases and low temperature reduce the expression of LAP at the cell surface

Weak bases such as chloroquine are known to induce a redistribution of LEP 100, a lysosomal membrane glycoprotein, to the cell surface (Lippincott-Schwartz and Fambrough, 1987). We determined the amount of cell surface LAP in cells that had been labelled and chased for 2 h each and then exposed for 30 min at 37°C to chloroquine, NH_4Cl, primaquine and monensin. Chloroquine (100 μM) and NH_4Cl (10 mM) reduced the amount of cell surface LAP to 60%, and primaquine (10 μM) to 35% of control. Monensin (10 μM) slightly increased the amount of cell surface LAP to 120% of control (Figure 11). The monensin-induced increase was consistently observed in four independent experiments. Incubation of BHK cells at reduced temperature led also to a redistribution of LAP. When cells were incubated for 2 h at 15, 20 or 25°C, the amount of cell surface LAP was decreased to about half of control levels (Figure 12).

Discussion

This study shows that LAP precursors are transported to dense lysosomes via the cell surface. The newly synthesized LAP is transferred from the endoplasmic reticulum via the trans-Golgi to the cell surface within 45 min. Cell surface associated LAP is rapidly internalized into a compartment from where it can recycle to the cell surface at $\geq$20°C. This compartment is considered to be part of the endosomal system. Essentially all LAP precursors that have passed the trans-Golgi and have not yet been delivered to dense lysosomes are part of this plasma membrane/endosome pool and participate in recycling. The estimates for the time required for completion of one round of transport vary between 6 min and $\leq$20 min, depending on the type of experimental approach (see Figures 5−8). The mean residence time of the LAP precursor in the plasma membrane/endosome pool is 5−6 h as calculated from the difference of the half-times required for transport to the cell surface (45 min, see Figure 3) and to dense lysosomes (6−7 h; S.Gottschalk *et al.*, 1989b). Thus, the LAP precursors recycle 15−50 times before they are delivered to dense lysosomes. Since at equilibrium ~4 times more LAP precursor is present in endosomes than at the cell surface, LAP is exposed at the cell surface only during one-fifth of the total cycle time. Our data were obtained with BHK cells expressing LAP at least two orders of magnitude above normal levels. Therefore, it cannot be excluded that the transport kinetics and the equilibrium distribution of LAP in cells that express lower levels of LAP are different. However, immunogold labelling of BHK cells expressing LAP levels ranging from 10- to 600-fold above normal demonstrated in all cells expression of LAP at the cell surface and no ob-

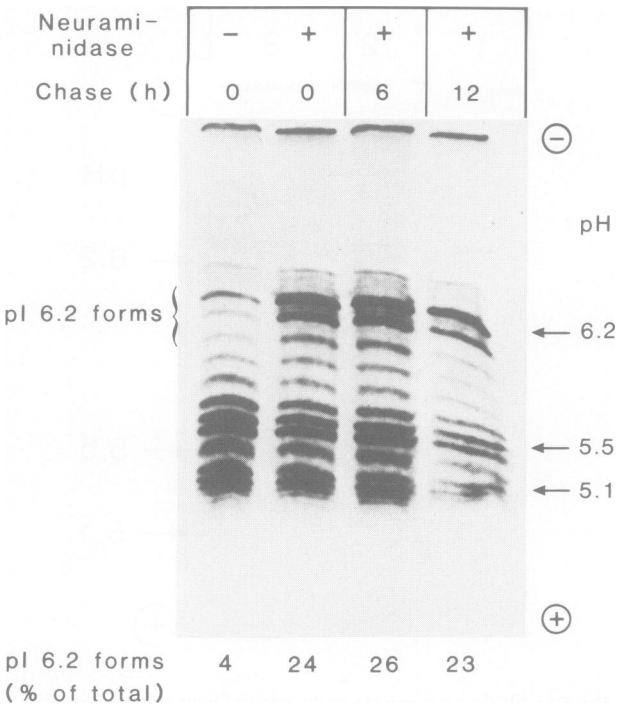

Fig. 10. LAP does not recycle to the site of sialyltransferase. Cells labelled and chased for 2 h were incubated at 4°C without (**lane 1**) or with neuraminidase (**lanes 2–4**). The cells were harvested directly (**lanes 1** and **2**) or after reculturing at 37°C for 6 h (**lane 3**) or 12 h (**lane 4**). LAP was immunoprecipitated from the Triton X-114 extract of cells and separated by IEF. For symbols and numbers below the lanes see Figures 1 and 2.

vious variation of its subcellular distribution (H. Geuze, unpublished). We assume therefore that, irrespective of the level of expression, LAP is transported to lysosomes via the cell surface.

The pathway from endosomes back to the cell surface is unlikely to involve passage on the Golgi/trans-Golgi network, since during recycling LAP was neither modified by Golgi mannosidase II nor by sialyltransferase. The effects of reduced temperature and weak bases such as chloroquine, NH_4Cl and primaquine on the distribution of LAP between the cell surface and intracellular membranes suggest that the rate constants for internalization and/or externalization of LAP are affected by these conditions. Reduced temperature and weak bases decrease the level of cell surface LAP.

In a separate study we have shown that the rapid internalization depends on a signal in the cytoplasmic tail of the LAP precursor and that tyrosine 413 is an essential part of this signal (C.Peters, unpublished). Tyrosine residues have been shown to be essential for the adaptor-mediated integration of endocytic receptors into clathrin-coated pits of the plasma membrane (Pearse, 1988; Glickman *et al.*, 1989). It is therefore likely that LAP utilizes clathrin-coated pits for internalization. This assumption is supported by the concentration of LAP in clathrin-coated membranes seen in immunoelectron microscopy (H.Geuze, unpublished). Deletion of the cytoplasmic tail or substitution of the tyrosine 413 by a phenylalanine impairs severely the internalization and results in the accumulation of the mutant LAP precursor at the cell surface (C.Peters, unpublished observation). Thus, the rapid signal-dependent internalization of LAP is critical for efficient routeing of LAP to dense lysosomes.

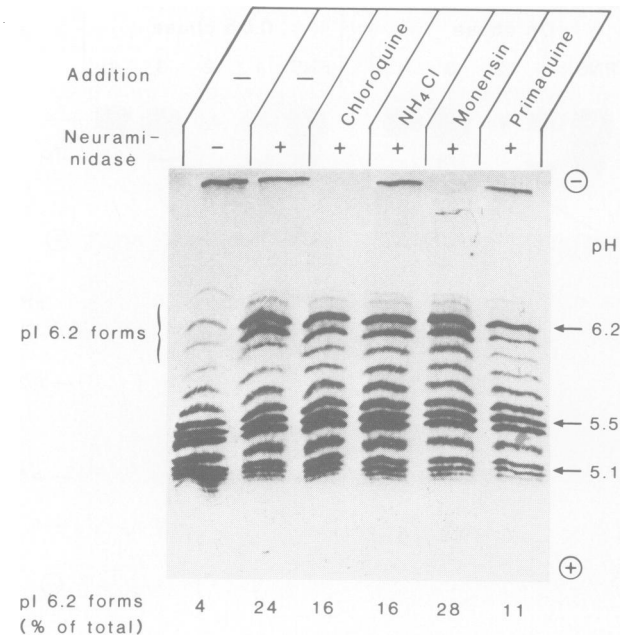

Fig. 11. Effect of weak bases and monensin on surface LAP. Cells labelled and chased for 2 h each were incubated for 0.5 h at 37°C in the absence (**lane 1**) or presence of 0.1 mM chloroquine (**lane 2**), 10 mM NH_4Cl (**lane 3**), 10 μM monensin (**lane 3**) or 10 μM primaquine (**lane 4**) and then treated at 4°C with neuraminidase. LAP was immunoprecipitated from the Triton X-114 extract of cells and separated by IEF. For symbols and numbers below the lanes see Figures 1 and 2.

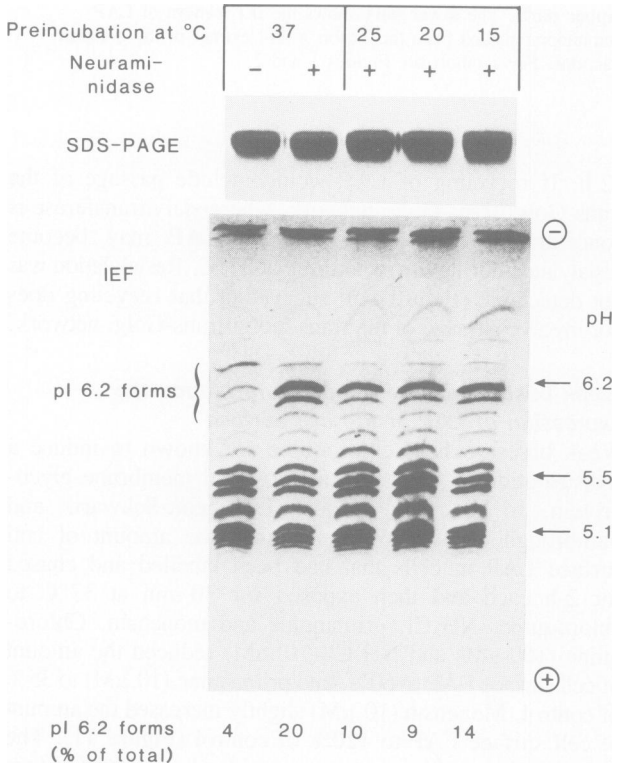

Fig. 12. Effect of temperature on surface LAP. Cells labelled and chased for 2 h were incubated for 2 h at the temperatures indicated above the lanes and then treated at 4°C with neuraminidase. LAP was immunoprecipitated from the Triton X-114 extract of cells and separated by IEF. For symbols and numbers below the lanes see Figures 1 and 2.

The transfer of LAP from endosomes to dense lysosomes is rather slow ($t_{1/2}$ 5–6 h). Nevertheless, the transfer is probably faster than for most of the endocytic receptors, with which LAP shares the rapid endocytosis and recycling. The transport rate of endocytic receptors from endosomes to dense lysosomes has not been determined directly. A rough estimate is provided by the half-lives of endocytic receptors, since these receptors are rapidly degraded after delivery to lysosomes. The half-lives of receptors for asialoglycoproteins (Schwartz and Rup, 1983), low-density lipoproteins (Goldstein *et al.*, 1985) and transferrin (Omary and Trowbridge, 1981; Weissmann *et al.*, 1986) range from 8 to 60 h. The faster transfer of LAP into dense lysosomes does not necessarily imply involvement of a specific signal. The quaternary structure or physicochemical properties of the polypeptides may be important factors determining the probabilities of a membrane protein exiting endosomes in the direction of the cell surface or the lysosomes. For example, cross-linking of recycling receptors by antibodies is commonly followed by directing the receptors to lysosomes (Gregoriou and Rees, 1984; Grunfeld, 1984; Weissmann *et al.*, 1986).

LAP that has been delivered to dense lysosomes does not recycle to endosomes at a rate detectable by our methods. If a significant backflow of membrane constituents from dense lysosomes to endosomes (or other light membranes) exists, as has been suggested (Draye *et al.*, 1988), LAP must become excluded from it. Proteolytic processing of LAP to a soluble polypeptide, which occurs in dense lysosomes, would be a means to prevent recycling. Proteolytic processing in dense lysosomes, however, is a slow process ($t_{1/2}$ 6–7 h; S.Gottschalk *et al.*, 1989b) and can therefore not account for the mechanism by which recycling of LAP to endosomes is prevented.

Several lysosomal membrane glycoproteins have been detected in low amounts at the cell surface, including endolyn-78 (Croze *et al.*, 1989) and LAP-100 (Lippincott-Schwartz and Fambrough, 1987), while others including Lamp-1, Lamp-2 (Chen *et al.*, 1985), lgp 110 and lgp 120 (Lewis *et al.*, 1985) were not detectable at the cell surface. The lysosomal membrane glycoproteins, for which data on the primary sequence are available, all contain a single tyrosine residue in their cytoplasmic tail of 10–11 residues (Chen *et al.*, 1988; Fambrough *et al.*, 1988; Fukuda *et al.*, 1988; Howe *et al.*, 1988; Viitala *et al.*, 1988). This offers the possibility that these lysosomal membrane glycoproteins also utilize a tyrosine-mediated internalization as a mechanism for rapid transfer to dense lysosomes. It will be of interest to see whether the transport of other lysosomal membrane glycoproteins indeed involves passage of the cell surface recycling between the cell surface and internal membranes and whether the biological functions of these proteins are connected to this pathway.

Materials and methods

BHK cells stably transfected with cDNA for human LAP (Pohlmann *et al.*, 1988) were subcloned. A clone (281-7) with 300-fold higher activity of LAP than BHK-21 cells was used for this study. Cells in 35 mm dishes were labelled with 0.74–7.4 MBq [^{35}S]methionine (≥24.6 TBq/mmol) as described (von Figura *et al.*, 1983; Lemansky *et al.*, 1985). During chase, the medium was supplemented with 0.25 mg/ml methionine. In experiments where the cells had been treated with neuraminidase, the chase medium was additionally supplemented with 0.1 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. Where indicated, 10 μg/ml swainsonine was present during the labelling and the chase.

Neuraminidase treatment

After metabolic labelling and chase the cells were washed twice with ice-cold PBS (10 mM sodium phosphate, pH 7.4, in 0.15 M NaCl) and incubated at 4°C for 1 h with 50 mU neuraminidase from *Vibrio cholerae* (Boehringer Mannheim) in 0.6 ml PBS. The neuraminidase was dialysed overnight against 50 mM Na-acetate, pH 5.5, containing 0.15 M NaCl and 9 mM $CaCl_2$. After washing twice with PBS containing 1% fetal calf serum and 0.1 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, the cells were either harvested or recultured at 37°C or incubated at 4°C with LAP antiserum.

Subcellular fractionation in Percoll gradients

The postnuclear supernatant obtained from cells that had been labelled and incubated with neuraminidase as indicated in the figure legends was fractionated by Percoll density centrifugation and analysed for marker enzymes and density as described (Lemansky *et al.*, 1984; Waheed *et al.*, 1988), except that the Percoll (Pharmacia) concentration was 20% (w/w) and that the sucrose cushion beneath the gradient was omitted. The gradient fractions were centrifuged at 300 000 *g* for 30 min. The membrane layer above the pellet of Percoll was collected and subjected to phase separation by Triton X-114 (see below).

Cell surface iodination

Cells grown to confluency in 35 mm dishes were incubated for 0.5 h at 0°C with 1 ml 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl, 10 mU lactoperoxidase (Sigma), 10 mU glucose oxidase (Boehringer Mannheim), 20 mM glucose and 0.56 MBq Na^{125}I (Amersham, carrier free) as described (Hubbard and Cohn, 1975).

Immunoprecipitation

Cells were harvested by scraping and solubilized in 1 ml of 10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 2% Triton X-114, 1 mM EDTA, 1 mM phenylethylsulfonyl fluoride and 5 mM iodoacetamide. After centrifugation of 30 min at 100 000 *g*, the supernatant was incubated for 5 min at 37°C and then centrifuged for 30 min at 500 *g* (Bodier, 1981). The Triton X-114 and the aqueous phase were adjusted to the original volume and Triton X-114 concentration. LAP was immunoprecipitated from the aqueous and the detergent phase as described (Waheed *et al.*, 1988). The immune complexes adsorbed to Immunoprecipitin (Bethesda Res. Lab.) were split. One-fifth was used for SDS–PAGE and four-fifths for IEF.

Immunoprecipitation of cell surface associated LAP

The cells were washed five times with ice-cold PBS and incubated for 4 h at 4°C with 10 μl LAP antiserum in 0.6 ml PBS. After washing twice with PBS containing 1% fetal calf serum, the cells were harvested by scraping. The cell pellet was suspended in 0.25 ml 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM phenylethylsulfonyl fluoride, 5 mM iodoacetate, and mixed with 0.2 ml 10 mM Na-phosphate, pH 7.4, containing 0.15 M NaCl, 0.5% SDS, 1% Triton X-100, 0.5% Na-deoxycholate and 2% bovine serum albumin. After sonication the homogenate was adjusted to 0.03% protamine sulfate, kept on ice for 10 min and centrifuged for 6 min at 12 000 *g*. The immune complexes were collected by incubation for 30 min with 2 μg Immunoprecipitin. The Immunoprecipitin was pretreated and the immune complexes were washed as described (Lemansky *et al.*, 1985). The remaining (intracellular) LAP was immunoprecipitated from the supernatant obtained after pelleting with Immunoprecipitin.

Electrophoresis

For SDS–PAGE, the immune complexes adsorbed to Immunoprecipitin were solubilized in the presence of 10 mM DTT and separated in 10% gels (Laemmli, 1970). For IEF the immune complexes adsorbed to Immunoprecipitin were washed twice with distilled water, solubilized by heating for 5 min at 95°C in 10 mM DTT, 0.5% SDS, lyophilized and then solubilized in lysis buffer and separated in 4% gels as described (O'Farrell, 1974). Radioactive polypeptides were visualized by fluorography and quantified by scintillation counting or densitometry. ^{14}C-Methylated protein standards were from New England Nuclear.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 236) and the Fonds der Chemischen Industrie.

References

Bodier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.

Chen,J.W., Murphy,T., Willingham,M., Pastan,J. and August,J.T. (1985) *J. Cell Biol.*, **101**, 85–95.

Chen,J.W., Cha,Y., Yuksel,K.U., Gracy,R.W. and August,J.T. (1988) *J. Biol. Chem.*, **263**, 8754–8758.

Croze,E., Ivanov,I.E., Kreibich,G., Adesnik,M., Sabatini,D.D. and Rosenfeld,M.G. (1989) *J. Cell Biol.*, **108**, 1597–1613.

Draye,J.P., Courtoy,P.J., Quintart,J. and Baudhuin,P. (1988) *J. Cell Biol.*, **107**, 2109–2115.

Fambrough,D.M., Takeyasu,K., Lippincott-Schwartz,J., Siegel,N.R. and Somerville,D. (1988) *J. Cell Biol.*, **106**, 61–67.

Fukuda,M., Viitala,J., Matteson,J. and Carlsson,S.R. (1988) *J. Biol. Chem.*, **263**, 18920–18928.

Gieselmann,V., Pohlmann,R., Hasilik,A. and von Figura,K. (1983) *J. Cell Biol.*, **97**, 1–5.

Glickman,J.N., Conibear,E. and Pearse,B.M.F. (1989) *EMBO J.*, **8**, 1041–1047.

Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russel,D.W. and Schneider,W.J. (1985) *Annu. Rev. Cell Biol.*, **1**, 1–39.

Gottschalk,S., Waheed,A. and von Figura,K. (1989a) *Biol. Chem. Hoppe-Seyler*, **370**, 75–80.

Gottschalk,S., Waheed,A., Schmidt,B., Laidler,P. and von Figura,K. (1989b) *EMBO J.*, **8**, 3215–3219.

Gregoriou,M. and Rees,A.R. (1984) *EMBO J.*, **3**, 929–937

Grunfeld,C. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2508–2511.

Howe,C.L., Granger,B.L., Hull,M., Green,S.A., Gabel,C.A., Helenius,A. and Mellman,I. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7577–7581.

Hubbard,A.L. and Cohn,Z.A. (1975) *J. Cell Biol.*, **64**, 438–460.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lemansky,P., Gieselmann,V., Hasilik,A. and von Figura,K. (1984) *J. Biol. Chem.*, **259**, 10129–10135.

Lemansky,P., Gieselmann,V., Hasilik,A. and von Figura,K. (1984) *J. Biol. Chem.*, **260**, 9023–9030.

Lewis,V., Green,S.A., Marsh,M., Vilko,P., Helenius,A. and Mellman,I. (1985) *J. Cell Biol.*, **100**, 1839–1847.

Lippincott-Schwartz,J. and Fambrough,D.M. (1987) *Cell*, **49**, 669–677.

Novikoff,P.M., Tulsiani,D.R.P., Touster,O., Yam,A. and Novikoff,A.B. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4364–4368.

O'Farrell,P.H. (1974) *J. Biol. Chem.*, **250**, 4007–4021.

Omary,M.B. and Trowbridge,J.S. (1981) *J. Biol. Chem.*, **256**, 12888–12892.

Pearse,B.M.F. (1988) *EMBO J.*, **7**, 3331–3336.

Pohlmann,R., Krentler,C., Schmidt,B., Schröder,W., Lorkowski,G., Cully,J., Mersmann,G., Geier,C., Waheed,A., Gottschalk,S., Grzeschik, H., Hasilik,A. and von Figura,K. (1988) *EMBO J.*, **7**, 2343–2350.

Roth,J., Faatjes,D.J., Lucoq,J.M., Weinstein,J. and Paulson,J.C. (1985) *Cell*, **43**, 287–295.

Schwartz,A.L. and Rup,D. (1983) *J. Biol. Chem.*, **258**, 11249–11255.

Tulsiani,D.R.P., Harris,T.M. and Touster,O. (1982) *J. Biol. Chem.*, **257**, 7936–7939.

Viitala,J., Carlsson,S.R., Sibert,P.D. and Fukuda,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3743–3747.

von Figura,K., Steckel,F. and Hasilik,A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6066–6070.

Waheed,A., Gottschalk,S., Hille,A., Krentler,C., Pohlmann,R., Braulke,T., Hauser,H., Geuze,H. and von Figura,K. (1988) *EMBO J.*, **7**, 2351–2358.

Weissmann,A.M., Klausner,R.D., Rao,K. and Itarford,J.B. (1986) *J. Cell Biol.*, **102**, 951–958.

Received on June 27,1989; revised on August 11, 1989