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Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells

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BHK cells transfected with human lysosomal acid phosphatase (LAP) cDNA (CT29) expressed 70-fold higher enzyme activities of acid phosphatase than non-transfected BHK cells. The CT29-LAP was synthesized in BHK cells as a heterogeneously glycosylated precursor that was tightly membrane associated. Transfer to the trans-Golgi was associated with a small increase in size (~7 kd) and partial processing of the oligosaccharides to complex type structures. CT29-LAP was transferred into lysosomes as shown by subcellular fractionation, immunofluorescence and immunoelectron microscopy. Lack of mannose-6-phosphate residues suggested that transport does not involve mannose-6-phosphate receptors. Part of the membrane-associated CT29-LAP was processed to a soluble form. The mechanism that converts CT29-LAP into a soluble form was sensitive to NH_4Cl, and reduced the size of the polypeptide by 7 kd. *In vitro* translation of CT29-derived cRNA in the presence of microsomal membranes yielded a CT29-LAP precursor that is protected from proteinase K except for a small peptide of ~2 kd. In combination with the sequence data available for LAP, these observations suggest that CT29-LAP is synthesized and transported to lysosomes as a transmembrane protein. In the lysosomes, CT29-LAP is released from the membrane by proteolytic cleavage, which removes a C-terminal peptide including the transmembrane domain and the cytosolic tail of 18 amino acids.

Key words: lysosomal membrane protein/protein transport

Introduction

The cDNA (CT29) that encodes human lysosomal acid phosphatase (LAP) predicts a polypeptide with a cleavable signal sequence at the N-terminus and a hydrophobic sequence close to the C-terminus (Pohlmann *et al.*, 1988). The latter may function as a transmembrane domain separating a short C-terminal tail of 18 amino acids at the cytosolic side from the remaining 87% of the polypeptide exposed at the luminal side of the membrane. Evidence for association of LAP with membranes comes from the observation that part of the LAP activity in human fibroblasts (Lemansky *et al.*, 1985; S.Gottschalk, unpublished data) and in COS cells transfected with CT29 (Pohlmann *et al.*, 1988) is partially membrane associated.

Here we report on the expression, transport and processing of CT29-LAP in baby hamster kidney (BHK) cells. CT29-LAP is synthesized as a transmembrane precursor and transported independent of mannose-6-phosphate receptors to lysosomes, where it is released from the lysosomal membrane by proteolytic cleavage.

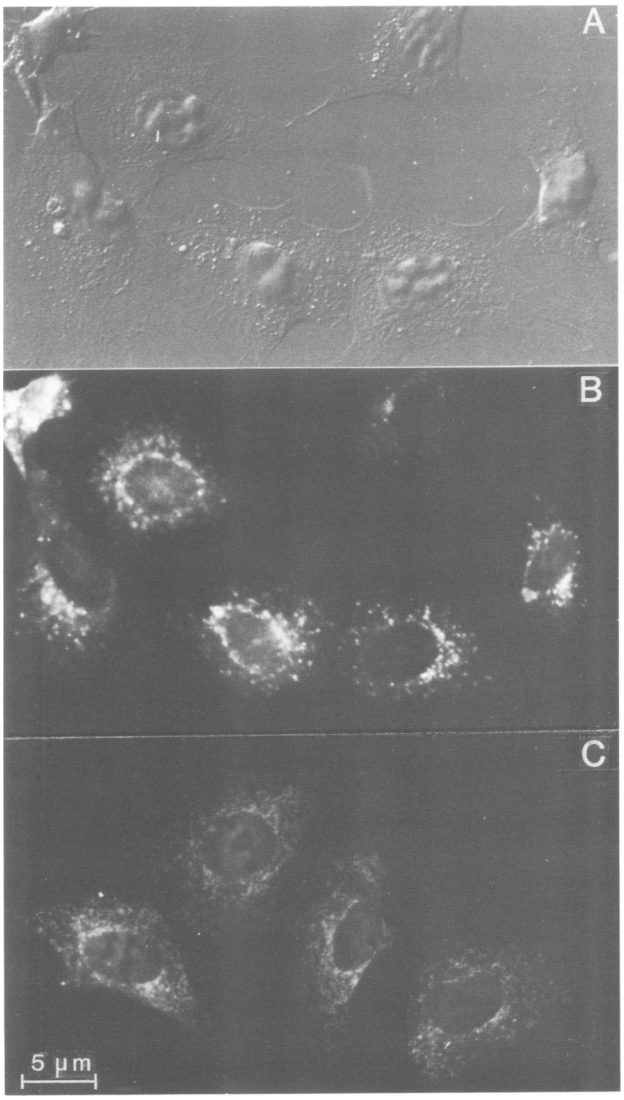

Fig. 1. Distribution of CT29-LAP in BHK cells. BHK-CT29 cells were incubated with rabbit anti-LAP (**A** and **B**) or rabbit anti-arylsulphatase B (**C**), followed by rhodamine conjugated anti-rabbit IgG. Interference contrast (**A**) and fluorescence (**B** and **C**) micrographs illustrate the distribution of lysosomes defined by arylsulphatase B (**C**) and the distribution of CT29-LAP (**B**).

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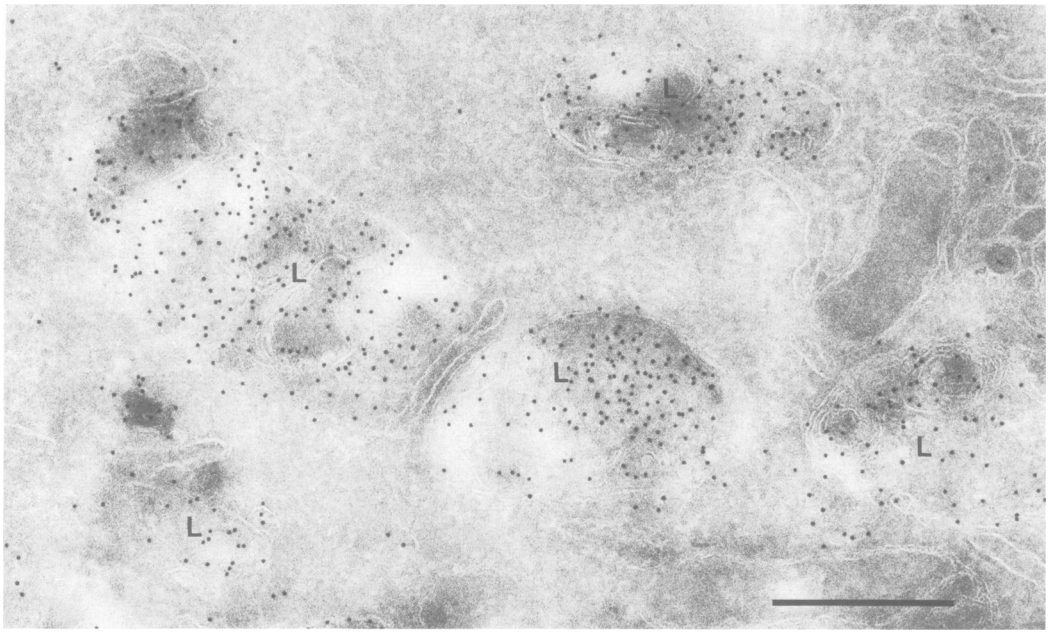

Fig. 2. Immunogold labelling of CT29-LAP in BHK cells. At least five lysosomes (L) can be seen, each of which showing abundant LAP labelling. The gold particles are membrane associated, but also occur over amorphous intra-lysosomal material. Magnification × 48 000; bar 0.5 μm.

Results

Stable expression of LAP in BHK cells

The cDNA of human LAP (CT29) was inserted into the expression vector pBEH for expression under control of the early simian virus 40 (SV40) promoter. pBEH-CT29 was cotransfected into BHK cells with pSV2pac, which confers resistance to puromycin to transfected cells. In stably transfected BHK cells (BHK-CT29), the activity of LAP (~0.5 U/mg cell protein) was ~70-fold higher than in non-transfected BHK cells and ~40-fold higher than in human skin fibroblasts.

Localization of CT29-LAP in lysosomes

Immunofluorescence and immunogold labelling were used to establish the localization of CT29-LAP in lysosomes of BHK-CT29 cells. Indirect immunofluorescence revealed a granular distribution of CT29-LAP preferentially in the perinuclear area (Figure 1B). No staining was found in BHK control cells (not shown). The distribution of CT29-LAP in transfected BHK cells was similar to that of two other lysosomal enzymes, arylsulphatase B (Figure 1C) and β-hexosaminidase (not shown). Immunogold labelling of cryosections of BHK-CT29 cells showed anti-LAP reactivity in association with the lysosomal membrane as well as in the lumen of lysosomes (Figure 2).

Association of CT29-LAP with membranes

About 40–50% of LAP activity could be extracted with 0.3 M NaCl. Solubilization of the remaining LAP activity required detergents such as Triton X-100. After solubilization of cells in Triton X-114 and separation into the detergent and aqueous phases, ~40% of LAP activity was recovered in the detergent phase. To analyse the molecular forms of LAP in the two phases, metabolically labelled BHK-CT29 cells were subjected to phase separation with Triton X-114 followed by immunoprecipitation. The Triton X-114 phase contained a 63-kd polypeptide and the aqueous phase a 52-kd

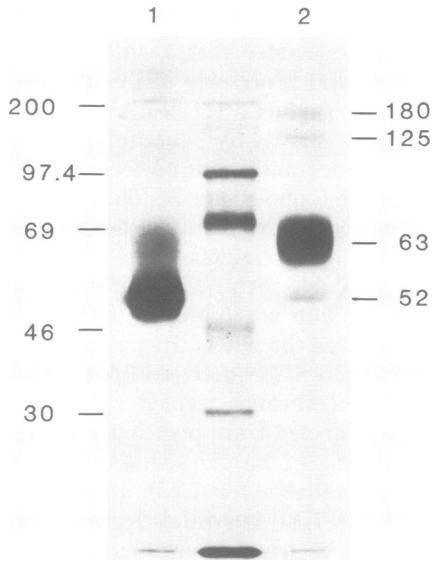

Fig. 3. Phase separation of CT29-LAP polypeptides in Triton X-114. BHK-CT29 cells were labelled for 2 h with [^{35}S]methionine and chased for 14 h. The cells were solubilized in Triton X-114 and phase-separated into the aqueous (**lane 1**) and detergent (**lane 2**) phase prior to precipitation of LAP. The mol. wt (kd) of the standards (middle lane) is given on the left and that of the CT29-LAP forms on the right.

polypeptide (Figure 3). The 63-kd polypeptide was accompanied by traces of ~125- and 180-kd polypeptides, which we believe to be di- and trimeric forms of the 63-kd polypeptide. No LAP polypeptides were detectable in the medium. Furthermore, labelling of endogenous LAP in non-transfected BHK cells was below the limit of detection (not shown). These results indicate that a part of CT29-LAP is associated with membranes and that the molecular forms of membrane-associated and soluble LAP differ.

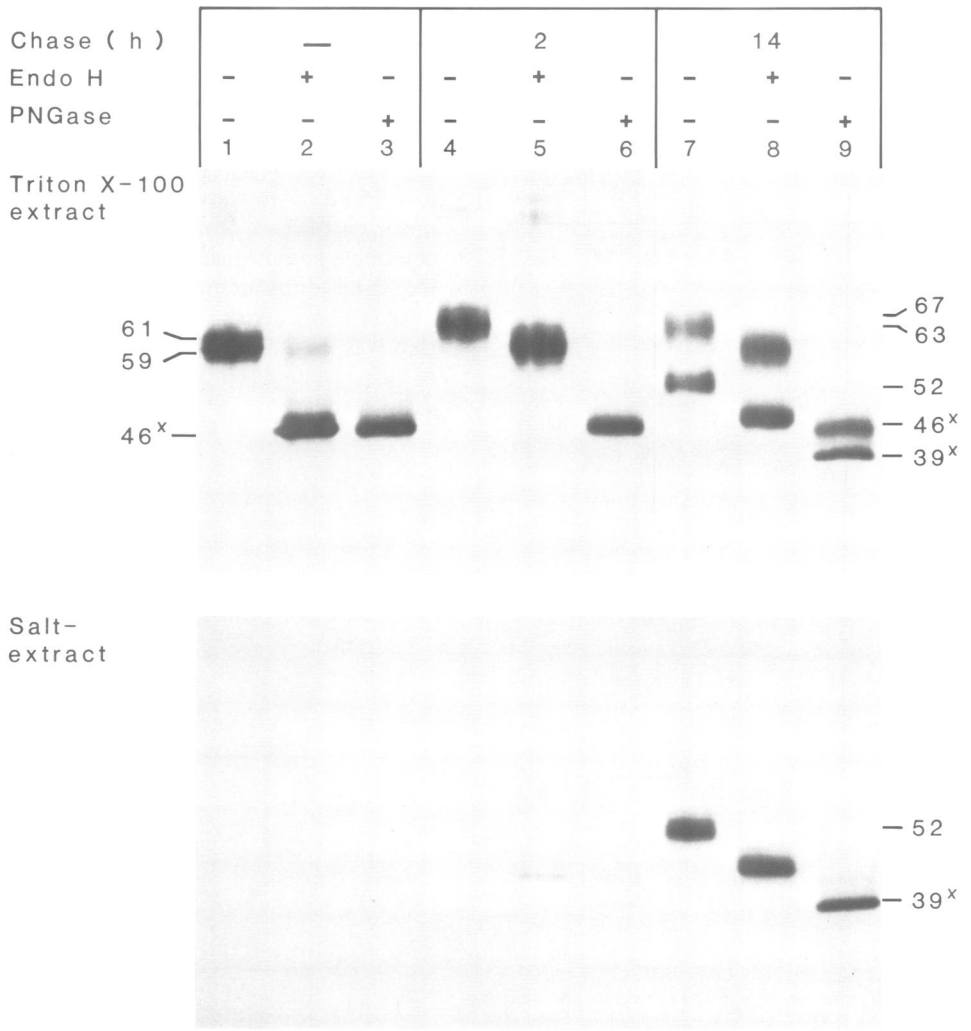

Fig. 4. Processing of CT29-LAP in BHK cells. BHK-CT29 cells were labelled for 30 min with [^{35}S]methionine and chased for 0, 2 and 14 h. The cells were sequentially extracted with salt (**lower part**) and Triton X-100 (**upper part**). CT29-LAP was immunoprecipitated and separated by SDS–PAGE before (**lanes 1, 4** and **7**) and after digestion with endoglucosaminidase H (**lanes 2, 5** and **8**) or PNGaseF (**lanes 3, 6** and **9**). The mol. wt of the glycosylated and PNGaseF treated (marked by 'x') forms are indicated.

Synthesis and processing of CT29-LAP in BHK cells

BHK-CT29 cells were labelled for 30 min with [^{35}S]-methionine and then chased for up to 14 h. After sequential extraction of cells with salt and detergent LAP was immunoprecipitated from the respective extracts (Figure 4). After 30 min labelling, a doublet of 59- and 61-kd polypeptides was detectable in the detergent extract. Deglycosylation with peptide: *N*-glycosidase F (PNGaseF) yielded a single 46-kd polypeptide indicating that the 59- and 61-kd precursors of CT29-LAP differ in *N*-glycosylation. Partial deglycosylation yielded seven distinct intermediates (Figure 5) suggesting that all of the eight potential *N*-glycosylation sites in CT29-LAP (Pohlmann *et al.*, 1988) are glycosylated.

The 59- and 61-kd precursors were converted within 2 h into a 67-kd polypeptide, which was partially resistant to endoglucosaminidase H and yielded a 46-kd product with PNGaseF (Figure 4). This indicates that the conversion of the 59–61-kd forms to 67-kd is due to oligosaccharide processing. To resolve the kinetics of oligosaccharide processing, the cells were labelled for 15 min and chased for up to 2 h (Figure 6). CT29-LAP forms containing oligosaccharides resistant to endoglucosaminidase H appeared after 15 min of chase. Within 30 min of chase, more than half of the CT29-LAP had acquired resistant oligosaccharides. This indicates that transport of CT29-LAP takes 30 min on average from the endoplasmic reticulum to the trans-Golgi, where oligosaccharides resistant to endoglucosaminidase H are generated (Kornfeld and Kornfeld, 1985).

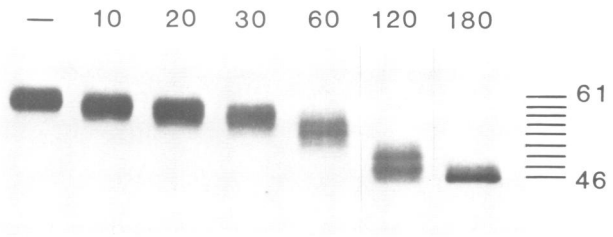

Fig. 5. Time course of endoglucosaminidase H digestion. CT29-LAP was immunoprecipitated from BHK-CT29 cells, which had been labelled for 15 min with [^{35}S]methionine and digested with endoglucosaminidase H for the time (min) indicated above the lanes. The nine discernible CT29-LAP forms are indicated.

Within 14 h of chase, the 67-kd form was converted into a 63-kd and a 52-kd form (Figure 4) (some gels reveal that the 63- and the 52-kd forms are in fact doublets). The 63-kd form was recovered in the membrane fraction and the 52-kd form mainly in the soluble fraction. The 52-kd form accounted for two thirds of the CT29-LAP polypeptides.

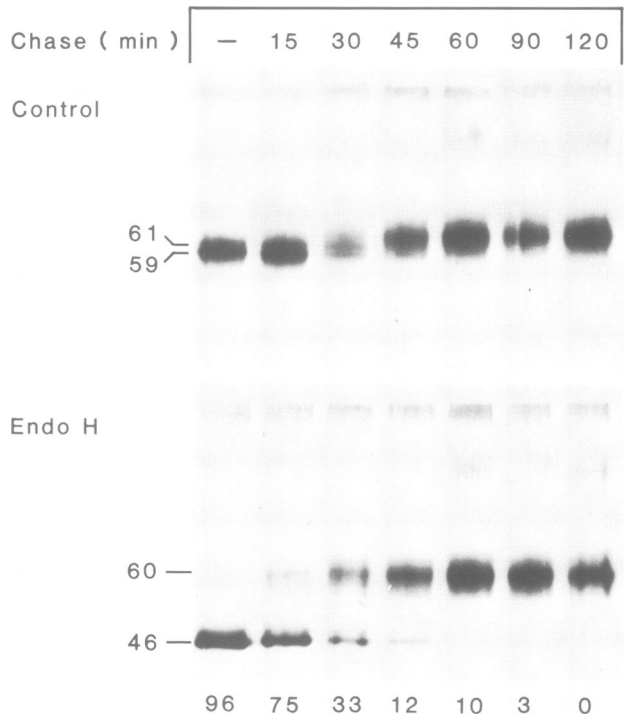

Fig. 6. Oligosaccharide processing in CT29-LAP. BHK-CT29 cells were labelled with [^{35}S]methionine for 15 min and chased for the time indicated above the lanes. The CT29-LAP immunoprecipitates were subjected to SDS−PAGE prior (**upper part**) and after treatment with endoglucosaminidase H (**lower part**). The mol. wt of the precursors (**upper part**) and the partially (60 kd) and fully deglycosylated (46 kd) CT29-LAP forms are indicated. The values at the bottom give the relative amount of the 46-kd form as a percentage of the total CT29-LAP.

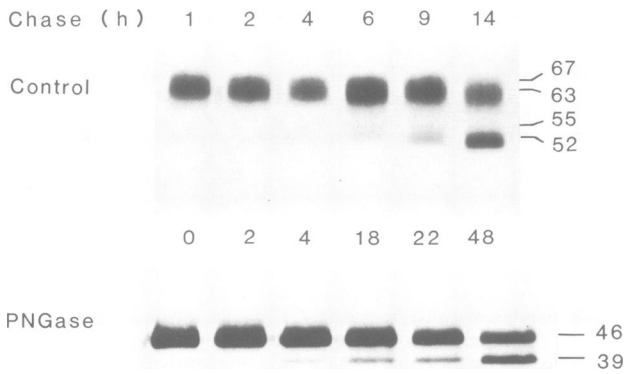

Fig. 7. Proteolytic processing of CT29-LAP. BHK-CT29 cells were labelled for 1 h with [^{35}S]methionine and chased for 1−14 h as indicated above the lanes. CT29-LAP was immunoprecipitated and subjected to SDS−PAGE before (**top**) and after (**bottom**) digestion with PNGaseF. The values below the top panel give the relative amount of the 52−55-kd forms as a percentage of the total CT29-LAP.

Treatment with endoglucosaminidase H and PNGaseF of the 63- and 52-kd polypeptides yielded products of 46 and 39 kd respectively. This suggests that processing of the 63-kd form to the 52-kd form involves removal of protein (~7 kd) and carbohydrate (~4 kd). An experiment with chase periods between 1 and 14 h revealed that the 67-kd form is processed to a 55-kd form, starting ~4 h after synthesis (Figure 7, upper part). This proteolytic processing occurred without detectable intermediates (Figure 7, lower part). The 67- and the 55-kd forms are processed, mostly between 9 and 14 h after synthesis, to the final 63- and 52-kd forms. The latter step was due to oligosaccharide processing, since the size of the deglycosylated polypeptides remained constant (Figure 7, lower part).

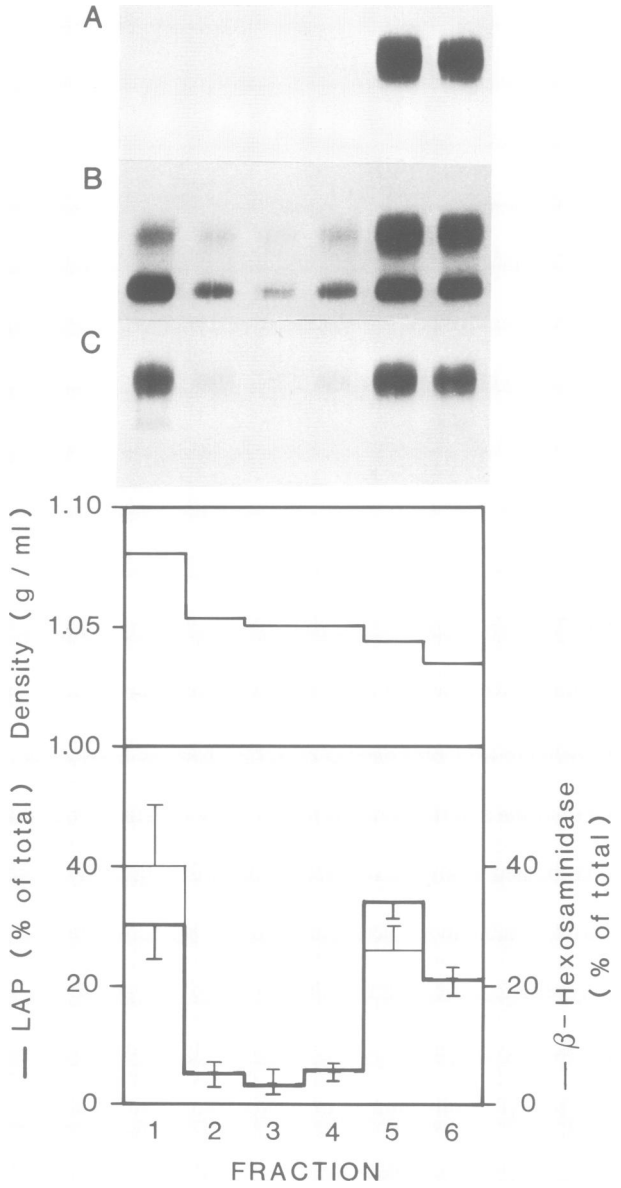

Fig. 8. Distribution of CT29-LAP in Percoll density gradients. BHK-CT29 cells were labelled for 2 h with [^{35}S]methionine and chased for 0 (**A**) or 14 h (**B** and **C**) in the absence (**B**) or presence (**C**) of 10 mM NH_4Cl. The postnuclear supernatant was subjected to Percoll density gradient centrifugation. CT29-LAP was immunoprecipitated from the gradient fractions. The density and the distribution of LAP and β-hexosaminidase activity of six independent experiments (standard deviation indicated by vertical bars) are given at the bottom.

Subcellular fractionation by Percoll density gradient centrifugation showed that initially (after labelling for 2 h) the 67-kd precursor is associated with light membranes (Figure 8A, fractions 5 and 6), which represent a mixture of endoplasmic reticulum, Golgi, plasma membrane, endosomes and light lysosomes (density profile and the distribution of LAP and β-hexosaminidase, a lysosomal marker, are shown in Figure 8, bottom). After a chase for 14 h the light membranes contained both the 63- and 52-kd forms, while the dense lysosomes were enriched in the 52-kd form (Figure 8B). When BHK-CT29 cells were grown for 3 days in the presence of 10 mM NH_4Cl, the 52-kd form was absent (Figure 9). Metabolic labelling of BHK-CT29 cells in the presence of NH_4Cl showed that the absence of the 52-kd form was not due to an enhanced secretion but to an inhibition of its formation (not shown). NH_4Cl caused accumulation of the membrane-associated 63-kd form of CT29-LAP in dense lysosomes (Figure 8C). This indicates that transport of CT29-LAP to dense lysosomes neither requires an acidic pH nor proteolytic processing.

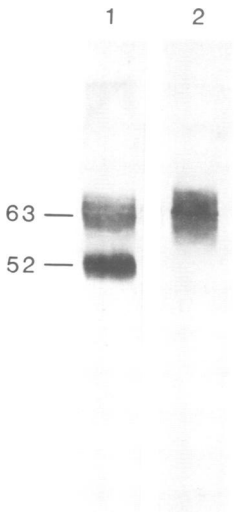

Fig. 9. Western blot of BHK-CT29 cells with anti-LAP. Extracts of cells that had been incubated for 3 days in absence (1) or presence (2) of 10 mM NH_4Cl were characterized by Western blotting (Conary *et al.*, 1987).

Phosphorylation of CT29-LAP

BHK-CT29 cells were labelled for 14 h in the presence of ^{32}P and extracted sequentially with salt and Triton X-100. The membrane-associated 63-kd form was found to be phosphorylated (Figure 10, lane 3), while the soluble 52-kd form did not contain phosphate (Figure 10, lane 1). Treatment with endoglucosaminidase H converted the phosphorylated 63-kd form into a phosphorylated 58-kd product without reducing the ^{32}P-label (Figure 10, lane 4). These results indicate that CT29-LAP polypeptides lack mannose-6-phosphate residues in high-mannose oligosaccharides, which are characteristic of soluble lysosomal enzymes, but contain phosphorylated amino acid(s). The latter are dephosphorylated or cleaved off concomitant to the processing of

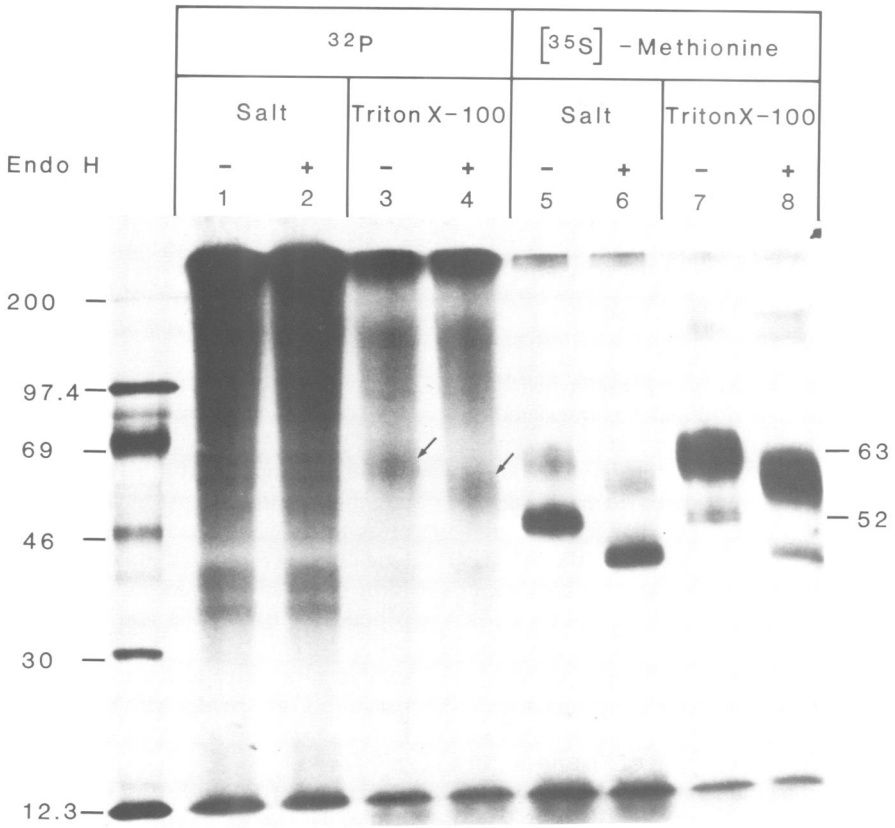

Fig. 10. Phosphorylation of CT29-LAP. BHK-CT29 cells were incubated for 14 h with ^{32}P (**lanes 1–4**) or [^{35}S]methionine (**lanes 5–8**). The cells were sequentially extracted with salt and Triton X-100. The CT29-LAP immunoprecipitates were incubated with buffer (−) or endoglucosaminidase H (+). The mol. wt (kd) of the standards (left margin) and LAP forms (right margin) is given. The phosphorylated forms of CT29-LAP are indicated by arrows. The gel was overexposed to reveal the faint ^{32}P-labelling of CT29-LAP.

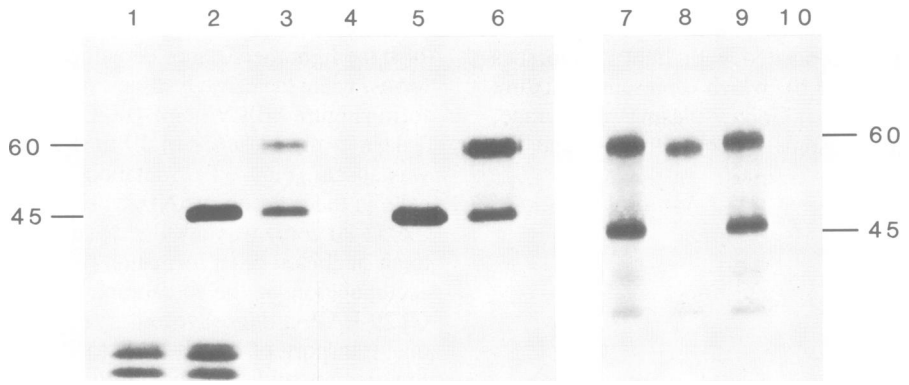

Fig. 11. *In vitro* synthesis of CT29-LAP. **Left.** CT29-cRNA was translated in a reticulocyte lysate in the absence (**lane 2**) and presence (**lane 3**) of dog pancreatic microsomes. **Lane 1** is a control without CT29-cRNA. **Lanes 1–3** show aliquots of the translation assays, **lanes 4–6** immunoprecipitates of the translation assays 1–3. **Right.** CT29-cRNA was translated in a reticulocyte lysate in the presence of microsomes (**lanes 7–10**) and digested with proteinase K in absence (**lane 8**) and presence (**lane 10**) of 0.1% Triton X-100. **Lanes 7** and **9** show a control incubated without proteinase K.

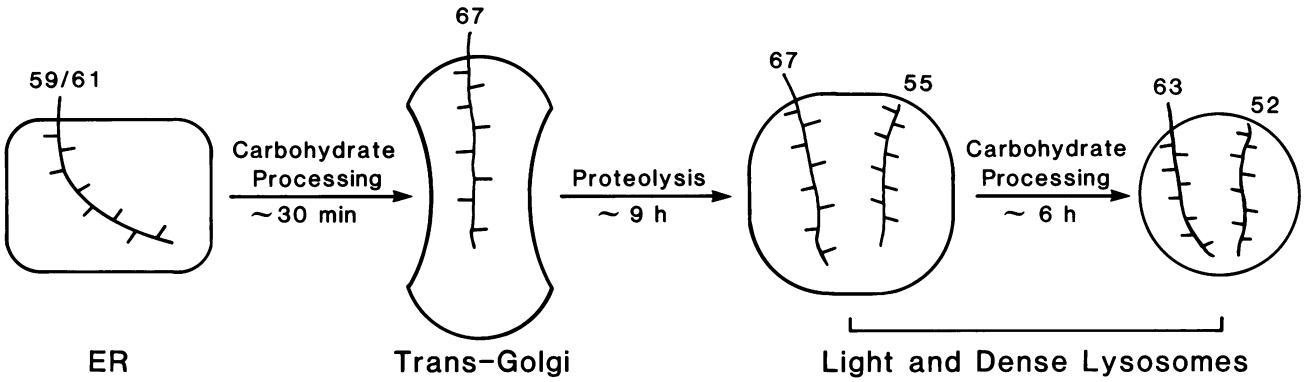

Fig. 12. Scheme for processing of CT29-LAP.

the membrane-associated 63-kd form to the soluble 52-kd form.

CT29-LAP is synthesized in vitro as a transmembrane protein with a small cytosolic tail

CT29-cDNA was transcribed *in vitro* and was translated in a reticulocyte lysate system. In the absence of microsomes, a 45-kd polypeptide was the major product (Figure 11, lanes 2 and 5). This polypeptide is considered to be the non-glycosylated precursor of CT29-LAP containing the signal sequence for which the nucleotide sequence predicts a size of 46.5 kd. In the presence of dog pancreatic microsomes, a major species of 60 kd was synthesized (Figure 11, lanes 3 and 6). This form is considered to be the glycosylated precursor of CT29-LAP lacking the signal sequence. Digestion of the translation mixture with proteinase K reduced the size of the translocated 60-kd precursor by 2 kd and resulted in loss of the non-glycosylated 45-kd precursor (Figure 11, lane 8). Digestion of the translation mixture with proteinase K in the presence of Triton X-100 led to complete loss of the 45- and 60-kd precursors (Figure 11, lane 10). These data indicate that CT29-LAP is synthesized as a transmembrane protein with a cytosolic tail of ~2 kd accessible to proteinase K.

Discussion

Synthesis as a transmembrane protein

The present results suggest that CT29-LAP is synthesized *in vitro* as a transmembrane protein with a short cytosolic tail. This had been predicted by the nucleotide sequence of the CT29 cDNA (Pohlmann *et al.*, 1988). The CT29-LAP precursor synthesized in BHK cells behaves as an integral membrane protein. At a later stage of its life cycle, presumably within the lysosomes, the CT29-LAP is proteolytically processed to a soluble form. The transmembrane form of CT29-LAP accounts for ~1/3 of the LAP polypeptides in BHK cells and for 40–50% of the catalytic activity. This shows that both CT29-LAP forms are catalytically active and that the transmembrane form may have a higher specific activity than the soluble CT29-LAP form.

Transport of CT29-LAP

Part of the oligosaccharides in CT29-LAP are processed into forms which are resistant to endoglucosaminidase H. This indicates that the pathway of CT29-LAP includes passage of the trans-Golgi. Between passage of the trans-Golgi (as a transmembrane protein) and the proteolytic processing, a

lag phase of ~4 h was observed. It is assumed that within this period the transmembrane precursor of CT29-LAP is segregated from membrane proteins with other destinations such as plasma membrane, and is transported to acidic organelles where proteolytic processing is initiated. The mechanism by which CT29-LAP is targeted into lysosomes differs from the mannose-6-phosphate receptor-dependent pathway of soluble lysosomal enzymes (for review see Kornfeld, 1986; von Figura and Hasilik, 1986). This is indicated by the absence of mannose-6-phosphate residues in CT29-LAP and the failure of antibiodies against the mannose-6-phosphate receptors to impair transport of CT29-LAP to lysosomes (S.Gottschalk, unpublished). Furthermore, the mechanism that ensures targeting of CT29-LAP to lysosomes is resistant to NH_4Cl. In contrast, the mannose-6-phosphate-receptor-dependent secregation of soluble lysosomal enzymes is impaired by receptor antibodies (Stein *et al.*, 1987a) and by weak bases such as NH_4Cl (Hasilik and Neufeld, 1980). The overexpression of CT29-LAP did not have an apparent effect on the sorting of soluble lysosomal enzymes. This was indicated by the comparable activities of soluble lysosomal enzymes in BHK-CT29 cells and in non-transfected BHK-21 cells.

Proteolytic processing

Similar to soluble lysosomal enzymes (for review see Skudlarek *et al.*, 1984; Hasilik and von Figura, 1985) CT29-LAP is proteolytically processed by a mechanism, which depends on an acidic pH and which occurs most likely after transfer into (light) lysosomes. The proteolytic processing renders CT29-LAP soluble. Therefore most likely the transmembrane domain and the cytosolic tail at the C-terminus are removed. Additional proteolytic processing at the N-terminus is unlikely since LAP purified from human placenta contains the N-terminus predicted by the CT29-cDNA (Pohlmann *et al.*, 1988). Proteolytic processing decreases the size of CT29-LAP by 11–12 kd, while its polypeptide is shortened only by 7 kd. This would suggest that a glycopeptide is cleaved off. Since the most C-terminal N-glycosylation site is 93 residues ahead of the C-terminus, such an event is unlikely. Using inhibitors of oligosaccharide trimming, we have observed that the carbohydrate has irregular effects on the electrophoretic mobility of CT29-LAP (S.Gottschalk, unpublished). Therefore, the simplest explanation is that proteolytic cleavage involves removal of a non-glycosylated C-terminal sequence and that the carboydrate causes anomalous behaviour of soluble and/or transmembrane CT29-LAP in SDS–PAGE. A scheme for the processing of CT29-LAP based on the findings in BHK cells is shown in Figure 12.

Comparison with LAP of human fibroblasts and with lysosomal membrane proteins

Multiple forms of CT29-LAP have been observed in human fibroblasts (Lemansky *et al.*, 1985; Waheed and van Etten, 1985). Part of these LAP-forms contained mannose-6-phosphate residues. Re-investigation of human skin fibroblasts showed that the LAP antiserum detects two differently glycosylated membrane-associated forms of 62 and 90 kd, which are deglycosylated to a single 46-kd polypeptide. In the salt extract of fibroblasts two precursors of 64- and 75-kd polypeptides are found. Phosphorylation of mannose residues was restricted to the soluble forms. The major phosphorylated form has a mol. wt of 54 kd and is secreted as the 64-kd form. In addition phosphorylated 110- and 112-kd forms are observed in the cells and the medium (S.Gottschalk, unpublished). The latter are likely to represent dimeric forms of the 54- and 64-kd polypeptides (Waheed and van Etten, 1985). Earlier studies have shown that the phosphorylated forms are transported to lysosomes in a mannose-6-phosphate-receptor-dependent manner (Lemansky *et al.*, 1985). The relationship of the CT29-LAP to the phosphorylated polypeptides, which were detected independently with antisera against LAP purified from human placenta (Lemansky *et al.*, 1985) and from human liver (Waheed and van Etten, 1985) is not clear. CT29-LAP resembles most closely the membrane-associated 62-kd forms in fobroblasts. Expression of CT29-cDNA in human skin fibroblasts and generation of antibodies against CT29-LAP may help to define the relationship between CT29-LAP and the various LAP forms in fibroblasts.

Several lysosomal membrane proteins of unknown function have been described recently (Chen *et al.*, 1985; Lewis *et al.*, 1985; Barriocanal *et al.*, 1986; Lippincott-Schwarz and Fambrough, 1986). In contrast to soluble lysosomal proteins, lysosomal membrane proteins including CT29-LAP have a relatively high content in complex-type oligosaccharides and lack mannose-6-phosphate residues. The structure of a lysosomal membrane glycoprotein, LEP 100, which shuttles between lysosomes, endosomes and plasma membrane has been described recently (Fambrough *et al.*, 1988). Both CT29-LAP and LEP 100 have in common a short C-terminal cytosolic tail of 18 and 11 amino acids respectively. It will be of interest to see whether this small cytosolic tail contains transport signals for lysosomal membrane proteins.

Materials and methods

Transfection of BHK cells

The *Eco*RI fragment of CT29-LAP (Pohlmann *et al.*, 1988) was subcloned into *Eco*RI-digested pBEH (Artelt *et al.*, 1988) by standard methods (Maniatis *et al.*, 1982). BHK cells (clone BHK-21) were maintained in Dulbecco's minimal essential medium with 10% fetal calf serum, and transfected with the calcium phosphate precipitate technique (Wigler *et al.*, 1977). The calcium phosphate precipitate for 5×10^5 BHK cells was made of 0.5 μg pBEH-CT29, 5 μg pSV2pac (Vara *et al.*, 1986) and 5 μg carrier DNA from BHK cells. Two days after transfection, the medium was supplemented with 5 μg/ml puromycin. Clones of stably transfected cells were pooled and assayed for L-tartrate inhibitable LAP activity (Waheed *et al.*, 1985).

Metabolic labelling and preparation of cell extracts

BHK cells in 35-mm dishes were labelled with 0.74–7.4 MBq [^{35}S]-methionine (≥24.6 TBq/mmol) or 55.5 MBq ^{32}P (carrier free) as described (von Figura *et al.*, 1983; Lemansky *et al.*, 1985). During chase, the medium was supplemented with 0.25 mg/ml methionine. The cells were harvested by scraping and extracts of cells and media were prepared as described (Lemansky *et al.*, 1985). In some experiments soluble and membrane-associated cellular proteins were separated either by Triton X-114 condensation (Stein *et al.*, 1987b) or by the following extraction: cells were sonicated in Tris/NaCl buffer [50 mM Tris–HCl pH 7.4, 0.3 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM iodoacetamide] and centrifuged for 30 min at 10^5 *g*. The extraction with Tris/NaCl buffer was repeated once and the supernatants were combined (soluble fraction). The pellet was extracted with 0.1% Triton X-100 in Tris/NaCl buffer and centrifuged as above (membrane-associated fraction).

Subcellular fractionation in Percoll gradients

Post-nuclear supernatants obtained from metabolically labelled cells were fractionated by Percoll density centrifugation and analysed as described

(Lemansky *et al.*, 1984), except that the Percoll (Pharmacia) concentration was 15% (w/w) and that the sucrose cushion beneath the gradient was omitted. The gradient fractions were adjusted to 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF and 5 mM iodoacetamide and centrifuged at 3×10^5 *g* for 30 min. The supernatants were used for immunoprecipitation.

In vitro transcription

CT29-LAP was inserted into the *Eco*RI site of the pGEM-1 plasmid (Promega Biotec). Ten micrograms of circular pGEM-1-CT29 were incubated with 50 U of SP6 polymerase (Boehringer) in 100 μl of 40 mM Tris−HCl pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 0.5 mM each of ATP, CTP and UTP, 0.2 mM [8-^{3}H]GTP (4.6 MBq/μmol), 1.0 mM 7-methyl-G(5′)ppp(5′)G (Pharmacia), and 0.2 U/μl RNasin (Boehringer) at 40°C for 60 min followed by treatment with 20 U of DNase I (Boehringer) at 37°C for 15 min. After extraction with phenol, chloroform and ether, RNA was precipitated out of 2 M ammonium acetate with 70% ethanol. The purified RNA was dissolved in sterile H_2O and stored at −20°C. Based on the incorporation of [^{3}H]GTP, ~5 μg of purified RNA was obtained per reaction. Full length transcripts were obtained as shown by PAGE in the presence of 8 M urea (Maniatis *et al.*, 1982).

In vitro translation

In vitro translation in reticulocyte lysate (New England Nuclear) was carried out according to the manufacturer's protocol in a 25-μl assay containing 50 ng of purified capped cRNA, 20 μCi of [^{35}S]methionine (51.8 TBq/mmol), 1 mM oxidized glutathione and, where indicated, 0.6 μl of dog pancreas membranes (New England Nuclear). For SDS−PAGE analysis, the reaction was stopped by addition of sample buffer. For immunoprecipitation of LAP, 3 vols of 1% Triton X-100, 0.5% Na-deoxycholate, 10 mM Na-phosphate, pH 7.4 and 0.15 M NaCl were added followed by sonication and centrifugation for 20 min at 48 000 *g* (4°C).

Portions (5 μl) of a translation mixture containing intact microsomes were incubated in the absence or presence of 0.3 mg/ml proteinase K (Boehringer) for 60 min on ice. Where indicated 0.3% Triton X-100 was present. The reaction was stopped by adjusting PMSF to 2 mg/ml. Samples were reduced with 20 mM DTT and alkylated with iodoacetamide (75 mM) and subjected to SDS−PAGE.

Immunoprecipitation

The extracts of cells and media, the Percoll density fractions and the translation assays were mixed with 0.8 vols of 10 mM Na-phosphate, pH 7.4 containing 0.15 M NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.5% SDS and 2 mg/ml bovine serum albumin. Cell extracts were adjusted to 0.03% protamine sulphate, kept on ice for 10 min and centrifuged at 12 000 *g* for 6 min. The samples were incubated for 1 h with pre-immune serum (1−2 μl) and 2−4 μg immunoprecipitin (Bethesda Research Laboratory) and centrifuged as above. The supernatants were incubated for 30 min with 2 μg immunoprecipitin and centrifuged for 1 h at 50 000 *g*. The LAP was immunoprecipitated from the supernatants as described (Lemansky *et al.*, 1985) except that incubation with antiserum was for 1 h only.

Treatment with endoglucosaminidase H or PNGaseF

Immune complexes were solubilized as described (Stein *et al.*, 1987b), except that DTT or β-mercaptoethanol were omitted. The supernatants were adjusted to 1 mM EDTA, 1 mM PMSF and 5 mM iodoacetamide and incubated for 20 h at 37°C in the absence or presence of 1 mU endoglucosaminidase H or 10 mU PNGaseF (both obtained form Boehringer).

Electrophoresis

The immune complexes were solubilized in the absence of reducing agents and subjected to SDS−PAGE in 10% gels (Laemmli, 1970) followed by fluorography (Bonner and Laskey, 1974). Radioactivity in polypeptides was quantified by scintillation counting (Waheed *et al.*, 1982). ^{14}C-Methylated protein standards were from New England Nuclear.

Indirect immunofluorescence and immunocytochemistry

All reagents were dissolved in 10 mM Na-phosphate, pH 7.4, 0.15 M NaCl (PBS). Cells grown on polylysine-coated glass coverslips were rinsed with PBS, fixed in 3% formaldehyde for 40 min, washed with PBS and 0.1 M glycine−Tris, pH 7.2 and permeabilized for 10 min by 0.3% Triton X-100. The cells were rinsed in 0.2% gelatine and incubated with antiserum against LAP or arylsulphatase B (Steckel *et al.*, 1983) for 1.5 h followed by tetramethylrhodamine-labelled anti-rabbit IgG (Dynatech) for 1 h.

BHK-CT29 cells and BHK control cells were fixed in a mixture of 1.0% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 0°C. The cells were embedded in gelatine (Geuze and Slot, 1980) to facilitate cryosectioning according to Tokuyashu and Singer (1976). The cryosections were indirectly immunolabelled with anti-LAP antibodies and 8-nm protein A gold particles. Sections were stained with uranyl acetate and embedded in methyl cellulose (Slot and Geuze, 1984).

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