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Mannose 6-Phosphate-independent Targeting of Cathepsin D to Lysosomes in HepG2 Cells*

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We have studied the role of *N*-linked oligosaccharides and proteolytic processing on the targeting of cathepsin D to the lysosomes in the human hepatoma cell line HepG2. In the presence of tunicamycin cathepsin D was synthesized as an unglycosylated 43-kDa proenzyme which was proteolytically processed via a 39-kDa intermediate to a 28-kDa mature form. Only a small portion was secreted into the culture medium. During intracellular transport the 43-kDa procathepsin D transiently became membrane-associated independently of binding to the mannose 6-phosphate receptor. Subcellular fractionation showed that unglycosylated cathepsin D was efficiently targeted to the lysosomes via intermediate compartments similar to the enzyme in control cells. The results show that in HepG2 cells processing and transport of cathepsin D to the lysosomes is independent of mannose 6-phosphate residues.

Inhibition of the proteolytic processing of 53-kDa procathepsin D by protease inhibitors caused this form to accumulate intracellularly. Subcellular fractionation revealed that the procathepsin D was transported to lysosomes, thereby losing its membrane association. Procathepsin D taken up by the mannose 6-phosphate receptor also transiently became membrane-associated, probably in the same compartment. We conclude that the mannose 6-phosphate-independent membrane-association is a transient and compartment-specific event in the transport of procathepsin D.

Cathepsin D is a major lysosomal aspartyl protease in mammalian cells. Like other soluble lysosomal enzymes, the glycoprotein is synthesized in the rough endoplasmic reticulum and subsequently provided with mannose-linked phosphate residues on the *N*-linked oligosaccharides. These mannose 6-phosphate (Man-6-P)¹ residues are then recognized by mannose phosphate receptors (MPR), of which two types have been identified. After binding to MPR in the Golgi

complex cathepsin D is targeted to a prelysosomal compartment where it is released from the MPR due to the acidic environment (Von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989).

In addition to this efficient receptor system, several lines of evidence indicate that MPR-independent targeting of soluble lysosomal enzymes to lysosomes exists. Generally, if the phosphorylation mechanism is defective, precursors of soluble lysosomal enzymes fail to be recognized by the MPR and consequently are secreted. This is observed in fibroblasts of patients suffering from I-cell disease (Hasilik and Neufeld, 1980). However, in some cell types and tissues of these patients, including hepatocytes, soluble lysosomal enzymes like cathepsin D are targeted to the lysosomes independent of the MPR (Owada and Neufeld, 1982; Neufeld and McKusik, 1983; Kornfeld, 1986; Nolan and Sly, 1989). In addition, transport of lysosomal membrane proteins to the lysosomes is unaffected in the absence of *N*-linked oligosaccharides, indicating their independence of MPR (Barriocanal *et al.*, 1986; Croze *et al.*, 1989). An intermediate position is observed in the case of the lysosomal enzyme glucocerebrosidase (Barranger and Ginns, 1989). Glucocerebrosidase is synthesized as a soluble protein but it becomes associated to cellular membranes during its transport to the lysosomes (Rijnboutt *et al.*, 1991). The protein does not acquire Man-6-P residues (Aerts *et al.*, 1988) and must therefore be targeted to the lysosomes independently of the MPR. Two separate studies have provided evidence that the proenzyme of cathepsin D can also be membrane-associated. Diment *et al.* (1988) found that procathepsin D is membrane-associated in the endosomes of rabbit macrophages and that this interaction is not mediated by Man-6-P. The membrane association disappeared concomitantly with proteolytic processing of the proenzyme. Similar results were obtained for cathepsin D and sphingolipid-activating protein in HepG2 cells where membrane association of the proenzymes occurred in or just distal to the Golgi complex (Rijnboutt *et al.*, 1991). The membrane association was detected after differential permeabilization of the cells with saponin, a cholesterol complexing agent (Schlösser and Wulff, 1969; Bangham and Horne, 1962). Differential permeabilization with saponin can selectively release secretory proteins but not membrane proteins (Wassler *et al.*, 1987; Strous and Van Kerkhof, 1989).

In this study we have used tunicamycin, an inhibitor of *N*-linked glycosylation, in combination with the differential permeabilization of cells with saponin. Our results with tunicamycin indicate that membrane association and transport of cathepsin D to the lysosomes was not dependent on the presence of *N*-linked oligosaccharides in HepG2 cells. In addition, we have used protease inhibitors to inhibit the proteolytic processing of cathepsin D in order to define the membrane association process as a sorting phenomenon (Von

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¹The abbreviations used are: Man-6-P, mannose 6-phosphate; MPR, mannose phosphate receptor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; E64: [*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amido-(4-guanidino)butane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

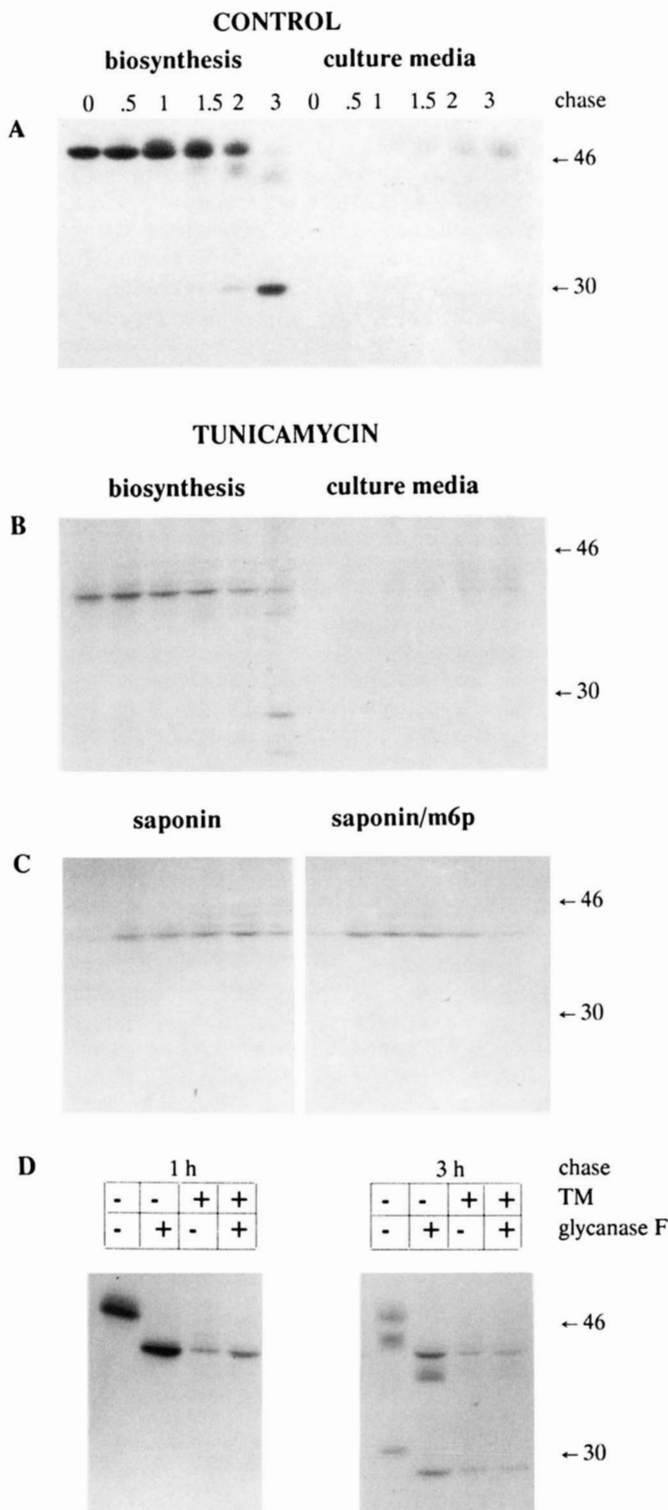


FIG. 1. Biosynthesis of cathepsin D in HepG2 cells in the absence or presence of tunicamycin. A, biosynthesis of cathepsin D. Cells were metabolically labeled with [³⁵S]methionine for 15 min and chased for the times indicated (h). Cells were immediately solubilized after the chase period and cathepsin D was immunoprecipitated from the lysates as well as the culture media and analyzed on SDS-PAGE. Relative molecular mass standards are indicated on the right. B, Cells were labeled as in A except that 4 h prior to and throughout the pulse-chase-labeling procedure, 10 μ g/ml tunicamycin was present. (Note that all metabolic forms of unglycosylated cathepsin D had lower molecular weights compared with cathepsin D from control cells.) C, experiment similar to that presented in B except that after the chase period the cells were differentially permeabilized with saponin in the absence (left) or presence (right) of Man-6-P. D,

Figura *et al.*, 1983; Hentze *et al.*, 1984; Steckel *et al.*, 1985; Gieselmann *et al.*, 1985; Hanewinkel *et al.*, 1987; Samarel *et al.*, 1989). We show that in the presence of the protease inhibitors E64 and pepstatin the Man-6-P-independent membrane association of procathepsin D was lost during the transport of procathepsin D to the lysosomes, indicating a compartment-specific process.

MATERIALS AND METHODS

Metabolic Labeling and Permeabilization of Cells—The human hepatoma cell line HepG2 (Knowles *et al.*, 1980) was cultured in monolayer as described previously (Rijnboutt *et al.*, 1991). Human foreskin fibroblasts were cultured and labeled identically to the HepG2 cells. HepG2 cells were grown on 35-mm Petri dishes, washed with phosphate-buffered saline (PBS) and preincubated in Eagle's minimal essential medium without methionine (Gibco Laboratories). Cells were then labeled for 15 min with 60 μ Ci/ml [³⁵S]methionine (800–1200 Ci/mmol, Radiochemical Center, Amersham, United Kingdom) and chased for various periods of time in 1 ml of the culture medium. In some experiments the culture medium was supplemented with 10 μ g/ml tunicamycin (Boehringer, Mannheim) or the protease inhibitors E64, leupeptin, and pepstatin, each at a final concentration of 100 μ M. Stock solutions of tunicamycin (1 mg/ml) were prepared in 0.01 M NaOH, leupeptin (10 mM) in water, pepstatin (10 mM) in dimethyl sulfoxide, and E64 (10 mM) in 50% ethanol. These solvents themselves had no effect on the targeting and maturation of cathepsin D. Metabolic labeling of the cells in the presence of tunicamycin or protease inhibitors was preceded by a preincubation of the cells with the reagent for 4 or 3 h, respectively, and all further incubations were done in the presence of the drug(s). For differential permeabilization (Strous and van Kerkhof, 1989; Rijnboutt *et al.*, 1991), cells were kept on ice, washed with ice-cold PBS, and incubated in 1 ml of PBS containing 2 mg/ml saponin (Merck) for 30 min on a rocking platform. After 30 min the saponin-containing medium was removed and cells were washed with PBS. Further washing or repeating the saponin incubation did not result in an additional release of the membrane-associated procathepsin D. The cells finally were solubilized in 1 ml of PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. Aliquots of the cell extracts and culture media were used for immunoprecipitation of cathepsin D as described previously (Rijnboutt *et al.*, 1991). The fluorograms of the gels were quantitated by scanning full lanes in an LKB Ultrascan XL enhanced laser densitometer.

Glycanase F Digestion—After immunoprecipitation, immune complexes were dissociated by incubation for 5 min at 90 °C in 100 μ l of 1% SDS. Samples were then divided into two aliquots. Digestion of immunoprecipitated cathepsin D with or without 0.3 units/incubation *N*-glycanase F (Boehringer, Mannheim) was performed for 18 h at 37 °C in sterile 0.1 M sodium citrate buffer, pH 6.0, containing 1 mM EDTA, 0.2% SDS, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Subsequently, samples were subjected to SDS-PAGE and fluorography.

Cell Fractionation and Sucrose Density Gradient Centrifugation—HepG2 cells were grown on 9-cm Petri dishes and pulse-chase-labeled for 10 min with [³⁵S]methionine as described above. Cells were washed with ice-cold PBS and harvested by scraping in 1 ml of buffer containing 10 mM HEPES, 15 mM KCl, and 1.5 mM MgCl₂. Cells were homogenized in 50 strokes in a Dounce homogenizer with a tight fitting pestle (Kontes Glass Co., Vineland, NJ). The homogenate was centrifuged for 5 min at 300 \times g to remove nuclei. 750 μ l of the postnuclear supernatant was layered on top of a linear gradient (11 ml, 0.7–1.6 M sucrose, 20 mM Tris, pH 7.5, and 1 mM EDTA). Intracellular compartments were separated by centrifugation in a Beckman ultracentrifuge at 100,000 \times g using a SW 41 rotor for 3 h at 4 °C. Subsequently, fractions were immunoprecipitated in the presence of 0.5% Triton X-100 and subjected to SDS-PAGE. For unlabeled cells, aliquots of the fractions were analyzed by Western blotting.

***N*-glycanase F digestion of immunoprecipitated cathepsin D.** At the two chase times indicated cathepsin D species synthesized in the absence or presence of 10 μ g/ml tunicamycin (TM) as in A and B were each digested with *N*-glycanase F as described under "Materials and Methods" and analyzed by SDS-PAGE.

RESULTS

Membrane Association Is Independent of N-Linked Glycosylation—In HepG2 cells (Fig. 1A) and human fibroblasts (Fig. 2) cathepsin D is synthesized as a 51-kDa precursor which is converted to the 53-kDa species within 1 h. Most of the 53-kDa species is then processed via the 44-kDa species to the mature 31-kDa form within 3 h. Only a small percentage of the 53-kDa procathepsin D is secreted. This sequence of events has been reported for several other cell types (Hasilik and Neufeld, 1980; Erickson *et al.*, 1981; Rosenfeld *et al.*, 1982; Gieselmann *et al.*, 1983). When fibroblasts were treated with tunicamycin, a drug which specifically inhibits N-linked glycosylation thus preventing phosphorylation of mannose residues, newly synthesized unglycosylated procathepsin D is completely secreted (Fig. 2), as was found for cathepsin D and other soluble lysosomal proenzymes (Von Figura *et al.*, 1979; Erickson *et al.*, 1981; Rosenfeld *et al.*, 1982; Imort *et al.*, 1983; Hanewinkel *et al.*, 1987; Nishimura *et al.*, 1988a, 1988b). This would be expected if Man-6-P is the only lysosomal targeting signal for cathepsin D. However, cathepsin D in HepG2 cells behaved differently. If these cells were labeled in the presence of tunicamycin the resulting 43-kDa precursor was normally processed into a 39-kDa intermediate within 2 h and into a 28-kDa "mature enzyme" after 3 h. Neither significant increase of secretion nor degradation of unglycosylated procathepsin D was observed in HepG2 cells (Fig. 1B). To determine whether the inhibition of N-linked glycosylation of cathepsin D in HepG2 cells was complete, we incubated cathepsin D synthesized in the presence of tunicamycin with N-glycanase F. This enzyme removes N-linked oligosaccharides from glycoproteins regardless of their complexity. No further decrease of the apparent molecular weight was detected (Fig. 1D). Cathepsin D, immunoprecipitated from control cells, could be deglycosylated to a species with an apparent molecular weight similar to the cathepsin D synthesized in the presence of tunicamycin (Fig. 1D). We conclude that tunicamycin completely inhibits the glycosylation of cathepsin D.

Since unglycosylated procathepsin D is unable to bind to the MPR, presumably another kind of membrane association is required to retain the unglycosylated proenzyme in intracellular compartments. In a previous study we established that during its intracellular transport 53-kDa procathepsin D becomes membrane-associated (Rijnboutt *et al.*, 1991). In these experiments we used low concentrations of the cholesterol-complexing agent saponin to differentially permeabilize HepG2 cells. Under these conditions soluble proteins were rinsed out completely, whereas proteins attached to mem-

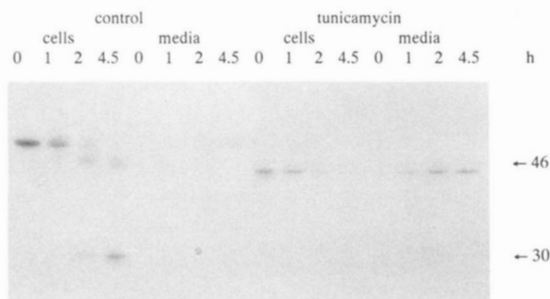


FIG. 2. **Biosynthesis of cathepsin D in fibroblasts.** Human skin fibroblasts were pulse-labeled with [³⁵S]methionine in the absence or the presence of 10 μ g/ml tunicamycin identically to HepG2 cells in Fig. 1. Cells were chased for the times indicated (h). After solubilization aliquots of the cells and the culture media were immunoprecipitated for cathepsin D and analyzed by SDS-PAGE.

branes were fully retained (Wassler *et al.*, 1987; Strous and Van Kerkhof, 1989; Rijnboutt *et al.*, 1991). To examine whether the observed membrane association of procathepsin D is related to the observed maturation of unglycosylated cathepsin D, pulse-chase-labeled cells were differentially permeabilized with saponin. As seen in Fig. 1C, the (unglycosylated) 43-kDa cathepsin D species became membrane-associated during the chase period as was previously found for the glycosylated procathepsin D. This association disappeared concomitantly with the appearance of the 39-kDa unglycosylated intermediate. If 10 mM Man-6-P was added to the saponin no additional procathepsin D was released, indicating that the membrane association of the 43-kDa procathepsin D was Man-6-P-independent. This concentration of Man-6-P has been shown to be sufficient to release all MPR-bound procathepsin D from the cells (Rijnboutt *et al.*, 1991).

We next studied whether unglycosylated cathepsin D can reach the lysosomes. Therefore, HepG2 cells were fractionated on sucrose density gradients after pulse-chase labeling in the absence or presence of tunicamycin. Cathepsin D was immunoprecipitated from the fractions and analyzed by SDS-PAGE. The distribution of the various species of cathepsin D from control cells after 3 h of chase shows that the 53-kDa intermediate was found in fractions 9–11, whereas the 44-kDa form was present in fractions of slightly higher densities (Fig. 3A). The 31-kDa mature form cofractionated with the lysosomal marker β -hexosaminidase near the bottom of the tube (fractions 4–9). The distribution of the completely unglycosylated mature cathepsin D, synthesized in the presence of 10 μ g/ml tunicamycin (Fig. 3C, fractions 4–9), shows that cathepsin D was targeted to the lysosomes independent of the presence of N-linked oligosaccharides. This is also illustrated in Fig. 3B, where intermediates of both normally glycosylated and unglycosylated cathepsin D are synthesized at the same time due to the use of an intermediate concentration of tunicamycin (5 μ g/ml). In this gradient related species of

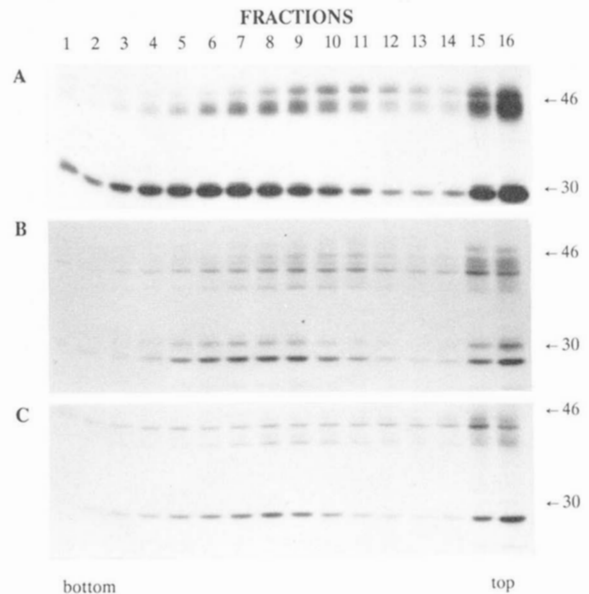


FIG. 3. **Subcellular fractionation of cathepsin D on linear sucrose density gradients.** HepG2 cells were labeled with [³⁵S]methionine as in Fig. 1 and chased for 3 h. In A no drug was added. In B and C cells were treated with 5 and 10 μ g/ml tunicamycin, respectively. The cells were then homogenized and fractionated by sucrose-density centrifugation. Subsequently, cathepsin D was immunoprecipitated from the fractions and subjected to SDS-PAGE. The enzymatic activity of galactosyltransferase (Strous and Berger, 1982), a Golgi marker enzyme, was present in fractions 7–9.

control and unglycosylated cathepsin D have the same distribution so that they probably use identical intracellular compartments while routed to the lysosomes. Using Western blot analysis with unlabeled cells, which were incubated in the presence of 10 $\mu\text{g}/\text{ml}$ tunicamycin for 7 h, the unglycosylated 28-kDa mature form cofractionated with the pre-existing 31-kDa mature form in fractions 6–9, indicating the 28-kDa form had been targeted to the lysosomes (not shown).

Effect of Protease Inhibitors on Membrane Association—Our previous study on normally glycosylated cathepsin D (Rijnboutt *et al.*, 1991) together with the present data obtained with tunicamycin (Fig. 1) suggests that the Man-6-P-independent membrane association of cathepsin D ends after proteolytic processing of the precursor proteins. To investigate this, HepG2 cells were pulse-chase-labeled in the presence of two protease inhibitors. In the presence of both E64, a specific and irreversible inhibitor of cysteine proteases, and pepstatin, an inhibitor of aspartyl proteases, the proteolytic processing of the 53-kDa procathepsin D was inhibited causing accumulation of procathepsin D intracellularly (Fig. 4B). Both protease inhibitors have previously been shown to inhibit proteolytic processing and degradation of lysosomal enzymes (Hentze *et al.*, 1984; Richardson *et al.*, 1988; Samarel *et al.*, 1989). Only after 3 h of chase a slight decrease to an unusual molecular mass of 47-kDa was detectable. Fig. 4B shows that the membrane association of the 53-kDa procathepsin D increased due to accumulation of this form as detected by differential permeabilization of the cells with saponin in the presence of Man-6-P. Approximately 20% of the 53-kDa procathepsin remained membrane-associated at each chase time after incubation with saponin in the presence of Man-6-P, as was estimated after fluorogram scanning. Concomitantly, the 47-kDa form had lost its membrane association. In control cells (Fig. 4A), the protease inhibitors had no effect on the amount of secretion of procathepsin D into the culture medium (Fig. 4B). Identical results were obtained when E64 was replaced by leupeptin, another inhib-

itor of cysteine proteases (data not shown).

The conversion of the 53-kDa procathepsin D to the 47-kDa intermediate form could be due to either oligosaccharide or proteolytic processing. To determine this cathepsin D, synthesized in the absence or the presence of protease inhibitors, was digested with glycanase F to remove all N-linked oligosaccharides. Both the 53- and the 47-kDa forms of cathepsin D synthesized in the presence of E64 and pepstatin were converted to the same form with an apparent molecular mass of 43 kDa (Fig. 5B). All cathepsin D forms from control cells detected after digestion with glycanase F were identical to those found in the tunicamycin experiment (compare Figs. 5A and 1D). Thus, the 47-kDa form which had accumulated in the presence of protease inhibitors has the same peptide backbone as the 53-kDa procathepsin D, and the difference in apparent molecular mass was due to modifications in the N-linked oligosaccharides.

To determine which compartments are involved in the processing and transport of the different metabolic forms of cathepsin D we performed subcellular fractionation of pulse-chase-labeled cells on sucrose gradients as in Fig. 3. Cells were pretreated with E64 and pepstatin as described above, pulse-labeled with [^{35}S]methionine for 30 min, and chased for 4 h, both in presence of the drugs. When cathepsin D was synthesized in the presence of E64 alone, an incomplete inhibition of the proteolytic processing was observed (Fig. 6B), showing the distribution of all metabolic forms in the same gradient. Biosynthesis in the presence of E64 did not change the distribution of the 53-kDa procathepsin D (Fig. 6B, fractions 9–12). The 47-kDa form of the procathepsin D codistributed with the 44-kDa intermediate form and the 31-kDa lysosomal form (fractions 4–7), indicating that the 47-kDa form had been translocated to the lysosomal compartment. If leupeptin was used instead of E64 a similar distribution of cathepsin D was obtained (not shown). The presence of E64 caused the localization of the 44- and 31-kDa forms to shift to a slightly higher density as compared with their distribution in control cells (Figs. 6A and 3A). In an identical experiment in which unlabeled cells were treated with E64 for 4 h, all of the pre-existing 31-kDa form, synthesized before E64 administration, behaved in a manner identical to that of

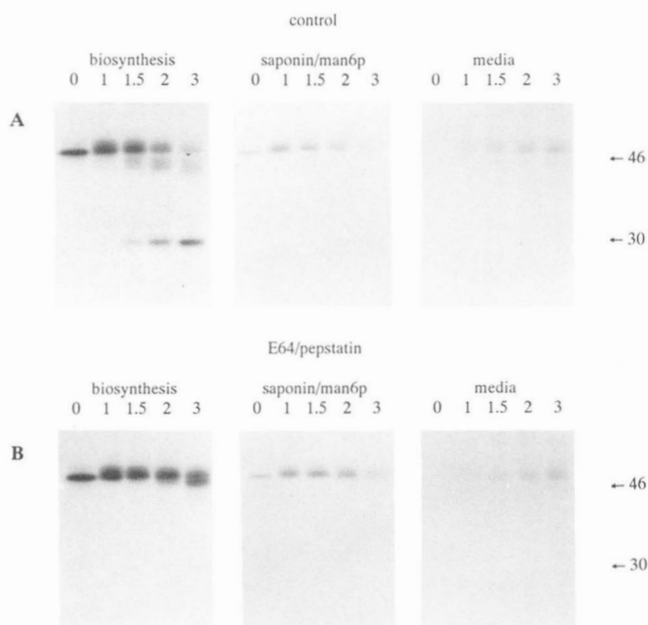


FIG. 4. Inhibition of the proteolytic processing of cathepsin D by E64 and pepstatin. A, control cells were pulse-chase-labeled for times indicated as in Fig. 1A. Differential permeabilization of the cells with saponin in the presence of Man-6-P was performed on ice as described under "Materials and Methods." B, as in A except that cells were preincubated for 3 h and pulse-chase-labeled in presence of E64 and pepstatin.

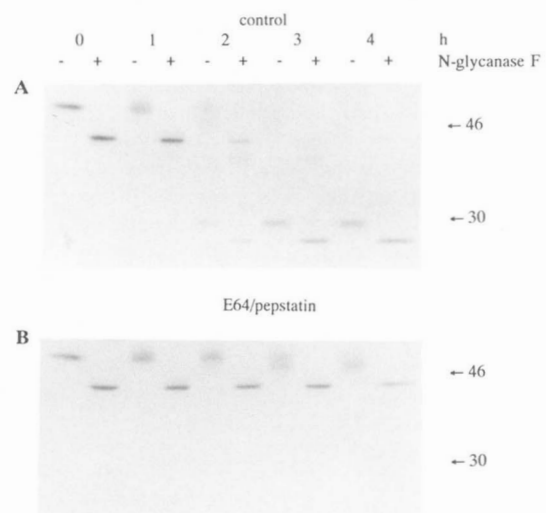


FIG. 5. N-Glycanase F digestion of cathepsin D synthesized in the presence of E64 and pepstatin. Immunoprecipitated cathepsin D from control cells (A) and from cells grown in the presence of 100 μM E64 and 100 μM pepstatin as in Fig. 4B (B) was digested with N-glycanase F as described under "Materials and Methods" and subsequently analyzed by SDS-PAGE.

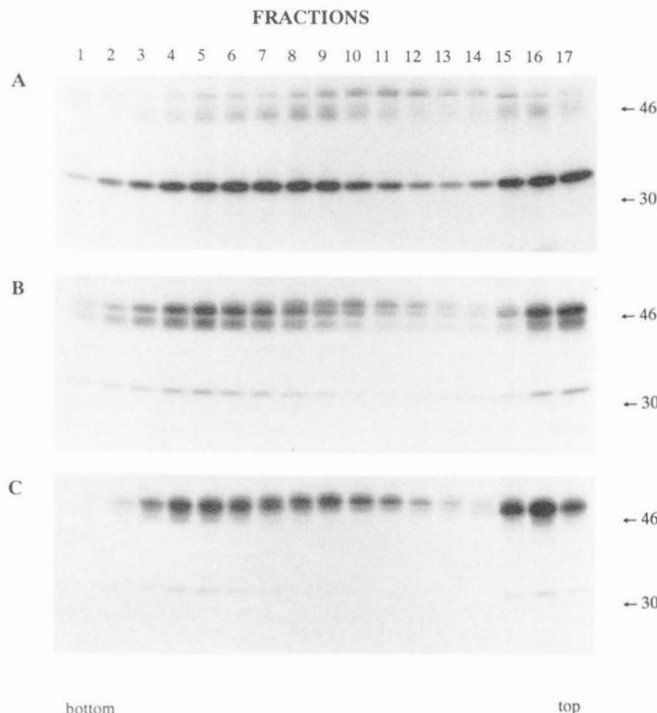


FIG. 6. Subcellular fractionation of cathepsin D on linear sucrose density gradients after inhibition of the proteolytic processing. HepG2 cells were preincubated for 3 h with or without protease inhibitors, labeled with [35 S]methionine as in Fig. 1, and chased for 4 h. In *A* no drug was added. In *B* and *C* cells were treated with 100 μ M E64 alone or 100 μ M E64 and 100 μ M pepstatin, respectively. The cells were then homogenized, and the homogenate was fractionated by centrifugation. Subsequently, cathepsin D was immunoprecipitated from the fractions and subjected to SDS-PAGE. The enzymatic activity of galactosyltransferase (Strous and Berger, 1982), a Golgi marker enzyme, was present in fractions 7–9.

the newly synthesized 31-kDa in Fig. 6*B* (data not shown). This result confirmed that E64 slightly increases the density of lysosomes, again demonstrating that the newly synthesized 47-, 44-, and 31-kDa forms were properly targeted to lysosomes. This experiment shows that the presence of E64 and pepstatin caused a complete inhibition of proteolytic processing of procathepsin D as seen before (Figs. 4*B* and 5*B*) but did not alter the distribution of the 53- and 47-kDa forms (Fig. 6*C*). From these fractionation studies it is clear that the procathepsin D is transported to the lysosomes in the absence of proteolytic processing. Its arrival in the lysosomes is monitored by the conversion of the molecular mass from 53 to 47 kDa due to modifications on the *N*-linked oligosaccharides. Thus, we conclude that MPR-independent membrane association is independent of removal of the propeptide and only occurs in a light density organelle.

Endocytosed Procathepsin D Becomes Membrane-associated—Finally we examined whether exogenously added procathepsin D also passes through a membrane association phase. [35 S]Methionine-labeled procathepsin D, synthesized and secreted by HepG2 cells grown in the presence of 10 mM NH_4Cl , was collected and added to unlabeled HepG2 cells (Fig. 7). Uptake was performed exclusively by the MPR, since addition of 10 mM Man-6-P to the uptake medium completely prevented uptake of [35 S]methionine-labeled cathepsin D (Fig. 7). The procathepsin D was efficiently processed to the 44-kDa intermediate form within 1 h and subsequently processed to the 31-kDa mature form after 2 h. Differential permeabilization with saponin showed that all of the 53-kDa form was associated with cellular membranes, whereas the

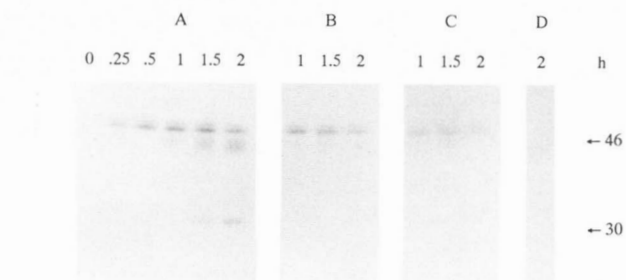


FIG. 7. Proteolytic processing and membrane association of endocytosed procathepsin D. HepG2 cells were incubated at 37 $^{\circ}\text{C}$ for times indicated with Eagle's minimal essential medium containing [35 S]-labeled procathepsin D, supplemented with 1 mg/ml bovine serum albumin. Cells were then washed and incubated on ice with PBS (*A*), PBS containing saponin (*B*), or PBS containing saponin and Man-6-P (*C*). In *D* uptake via the MPR was prevented by addition of 5 mM Man-6-P to the uptake medium. Subsequently, cells were washed with PBS and lysed in PBS-1% Triton X-100. Aliquots were immunoprecipitated for cathepsin D and analyzed by SDS-PAGE.

44- and 31-kDa forms could be completely rinsed out during the incubation with saponin. Addition of 10 mM Man-6-P to the saponin solution caused only part of the membrane associated material to be rinsed out. Surprisingly, part of the 53-kDa procathepsin D had become membrane-associated independently of Man-6-P. This indicated that the compartment in which the Man-6-P-independent membrane association of newly synthesized lysosomal enzymes occurs is also accessible to procathepsin D, which had been endocytosed by the MPR.

DISCUSSION

Several studies have shown that in most cells in the presence of tunicamycin (Von Figura *et al.*, 1979; Erickson *et al.*, 1981; Rosenfeld *et al.*, 1982; Imort *et al.*, 1983; Nishimura *et al.*, 1988a, 1988b) or in the presence of weak bases (Wiesmann *et al.*, 1975; Hasilik and Neufeld, 1980; Gonzalez-Noriega *et al.*, 1980; Dean *et al.*, 1984) all newly synthesized soluble lysosomal enzymes, including cathepsin D, are secreted into the culture medium without proteolytic processing. Our results show that in HepG2 cells unglycosylated cathepsin D is targeted to the lysosomes instead of being secreted. Therefore, we conclude that HepG2 cells possess an alternative targeting system for cathepsin D, which is not dependent on the presence of Man-6-P residues. This alternative transport mechanism probably acts in the same compartments as the MPR as indicated by the subcellular fractionation experiment. Proteolytic processing of the unglycosylated cathepsin D is as efficient as the processing of cathepsin D in control cells. This alternative targeting system has sufficient capacity for all newly synthesized cathepsin D to arrive in the lysosomes under conditions where targeting by MPR has been eliminated.

During the intracellular transport the unglycosylated procathepsin D becomes membrane-associated. This membrane association is likely part of the alternate targeting system for cathepsin D. We previously reported that the proenzyme for glycosylated cathepsin D, but also the precursors for glucocerebrosidase and sphingolipid activating protein, are initially present as soluble proteins in the rough endoplasmic reticulum but become membrane-associated as they arrive in the Golgi complex (Rijnboutt *et al.*, 1991). The mechanism involved for the Man-6-P-independent membrane association is unclear. To study the nature of the interaction we have tried to find conditions in which dissociation of procathepsin D from the membranes would occur by changing the conditions for semipermeabilization of the cells. Procathepsin D

was released from the membranes neither by incubation at pH values between 4 and 9, nor at ionic strengths varying between 0.05 and 1 M NaCl, nor using combinations of low pH and high ionic strength.² Only the detergent Triton X-100, which solubilizes membranes completely, could release membrane-associated cathepsin D. Because the membrane association responsible for sorting soluble lysosomal enzymes is operative in a few distinctive cell types, it is likely that these cells contain a specific class of membrane proteins or lipids used for this sorting in a post-Golgi compartment. The spatial conformation of the procathepsin D could also be of importance with respect to the membranes association. Diment *et al.* (1988) reported that the membrane association observed for procathepsin D in rabbit macrophages is present in endosomes and disappears upon proteolytic processing in a later prelysosomal compartment. This indicated that the peptide fragment which is proteolytically removed from 53-kDa procathepsin D to form the 44-kDa form was probably involved in the membrane interaction. Our experiments with protease inhibitors show that proteolytic processing is not a prerequisite for the dissociation of procathepsin D from the membranes, since the 47-kDa form was not membrane-associated. Therefore, the transient membrane association rather than proteolytic processing of procathepsin D or the presence of the propeptide probably plays a role in the targeting to lysosomes. Several other studies have indicated that the transport of lysosomal proenzymes to lysosomes is not dependent on proteolytic processing (Oude Elferink *et al.*, 1985; Samarel *et al.*, 1989). In contrast, in *Dictyostelium discoideum* (Richardson *et al.*, 1989) and *Xenopus* oocytes (Opresko and Karpf, 1987) inhibition of the proteolytic processing causes lysosomal proenzymes to accumulate prelysosomally.

Subcellular fractionation showed that both Man-6-P-dependent and -independent membrane associations are restricted to the same light density compartments (Rijnboutt *et al.*, 1991). The only membrane-associated form of cathepsin D fractionates in a distinct peak, suggesting a single compartment. The non-membrane-associated procathepsin D is probably secreted from this compartment. The biosynthetic studies indicate that the compartment is reached within 30 min by newly synthesized procathepsin D. Therefore, it is likely that the mannose 6-phosphate-independent membrane association starts in the Golgi-complex and is maintained until the procathepsin D reaches a prelysosomal compartment. Cathepsin D dissociates from the membrane before the degradation of the *N*-linked oligosaccharides starts, *i.e.* probably before entering the lysosome. Procathepsin D, taken up via MPR, becomes membrane-associated independently of the MPR, suggesting that the compartment in which newly synthesized cathepsin D becomes membrane-associated is also part of the endocytic pathway. This was also indicated in the studies of Diment *et al.* (1988) which showed that the membrane-associated procathepsin D is accessible to lactoperoxidase. Taken together, these observations suggest a compartment which has the characteristics of the *trans*-Golgi-reticulum or endosomes (Geuze and Morr , 1991).

The alternate targeting system present in HepG2 cells might represent the system responsible for transport of soluble lysosomal enzymes to lysosomes in tissues of patients with I-cell disease. This disorder is caused by a deficiency of phosphotransferase activity which results in an inability to synthesize the Man-6-P recognition signal. However, some tissues of patients with I-cell disease contain normal levels of soluble lysosomal enzymes, although the phosphotransferase

is absent in these tissues. This indicates the existence of an alternative targeting mechanism, different from the MPR system in these tissues (Owada and Neufeld, 1982; Neufeld and McKusik, 1983; Nolan and Sly, 1989; Kornfeld, 1986). Furthermore, the alternative mechanism of lysosomal enzyme targeting in HepG2 cells is reminiscent of that found in *D. discoideum*, where a mannose-phosphate-independent membrane association of lysosomal proenzymes is involved in transport to lysosomes (Mierendorf *et al.*, 1985; Cardelli *et al.*, 1986). Similar to our observations in HepG2 cells, the membrane association in the slime mold disappeared upon proteolytic processing (Richardson *et al.*, 1989; Cardelli *et al.*, 1989). However, when the proteolytic processing of lysosomal enzymes was prohibited with protease inhibitors, the proenzymes were secreted instead of being targeted to lysosomes as is shown in our present study (Richardson *et al.*, 1989). These authors have suggested that this is due to an overloading of a putative receptor for lysosomal targeting in *D. discoideum*, a process which apparently does not occur in HepG2 cells.

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