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Lysosomal Cysteine Protease, Cathepsin B, Is Targeted to Lysosomes by the Mannose 6-Phosphate–Independent Pathway in Rat Hepatocytes: Site-Specific Phosphorylation in Oligosaccharides of the Proregion[1]

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Cathepsin B, a lysosomal cysteine protease, is synthesized as a glycoprotein with two *N*-linked oligosaccharide chains, one of which is in the propeptide region while the other is in the mature region. When cultured rat hepatocytes were labeled with [^{32}P]phosphate, ^{32}P-labeled cathepsin B was immunoprecipitated only in the proform from cell lysates and medium. Either Endo H or alkaline phosphatase treatment of ^{32}P-labeled procathepsin B demonstrated the acquisition of a mannose 6-phosphate (Man 6-P) residue on high mannose type oligosaccharides. To identify the site of phosphorylation, immunoisolated ^{35}S- or ^{32}P-labeled procathepsin B was incubated with purified lysosomal cathepsin D, since cathepsin D cleaves 48 amino acid residues from the N-terminus of procathepsin B, in which one *N*-linked oligosaccharide chain was also included [Kawabata, T. *et al.* (1993) *J. Biochem.* 113, 389–394]. Treatment of intracellular ^{35}S-labeled procathepsin B with a molecular mass of 39-kDa with cathepsin D resulted in the production of the 31-kDa intermediate form, but the ^{32}P-label incorporated into procathepsin B disappeared after treatment with cathepsin D. These results indicate that the phosphorylation of procathepsin B is restricted to an oligosaccharide chain present in the propeptide region. Interestingly, cathepsin B sorting to lysosomes was not inhibited by NH_4Cl treatment and about 90% of the intracellular procathepsin B initially phosphorylated was secreted into the medium without being dephosphorylated intracellularly, and did not bind significantly to cation-independent-Man 6-P receptor, suggesting the failure of Man 6-P–dependent transport of procathepsin B to lysosomes. Additionally, about 50% of the newly synthesized ^{35}S-labeled cathepsin B was retained in the cells in mature forms consisting of a 29-kDa single chain form and a 24-kDa two chain form, while part of the procathepsin B was associated with membranes in a Man 6-P–independent manner. Taken together, these results show that in rat hepatocytes, cathepsin B is targeted to lysosomes by an alternative mechanism(s) other than the Man 6-P–dependent pathway.

Key words: cathepsin B, hepatocyte, lysosome, mannose 6-phosphate, protein sorting.

In mammalian cells, the targeting of newly synthesized soluble lysosomal enzymes depends on the presence of mannose 6-phosphate (Man 6-P) residues in their oligosaccharide side chains and their recognition by two Man 6-P–specific receptors (MPR), cation-dependent-MPR (CD-MPR) and cation-independent-MPR (CI-MPR), which are responsible for sorting soluble lysosomal enzymes from the secretory pathway (*1*, *2*). This recognition marker is synthesized in a two-step reaction. First, UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine 1-phosphotransferase (phosphotransferase) selectively transfers *N*-acetylglucosamine 1-phosphate to specific mannose residues within a high mannose type unit. In a second step, the *N*-acetylglucosamine residues are removed by *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase to generate Man 6-P monoesters, which are specifically recognized by MPRs present in the Golgi. The receptor-ligand complexes are subsequently transported to a prelysosomal/endosomal compartment where the lysosomal enzymes are dissociated from the receptors due to the acidic environment and delivered to lysosomes.

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Abbreviations Man 6-P, mannose 6-phosphate; ICD, I-cell disease; MPR, mannose 6-phosphate receptor; BFA, brefeldin A, Endo H, endo-β-*N*-acetylglucosaminidase H, PNGase F, peptide-*N*-glycosidase F, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, CI-MPR, cation-independent-MPR; CD-MPR, cation-dependent-MPR; TGN, *trans*-Golgi network; phosphotransferase, UDP-GlcNAc: lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase.

Recent gene targeting experiments in mice have further demonstrated the importance of MPRs for the proper sorting of lysosomal enzymes (*3–6*). Nevertheless, the existence of a Man 6-P–independent pathway has also been described. Earlier studies of I-cell disease (ICD) indicated that a Man 6-P–independent mechanism could be involved in the transport of lysosomal enzymes to lysosomes in some tissues and cell types (*7*, *8*). Fibroblasts from patients with ICD hypersecrete lysosomal enzymes as a consequence of defects in the Man 6-P generating enzyme, phosphotransferase (*9*, *10*); however, hepatocytes, Kupffer cells, and leukocytes in ICD patients are also deficient in phosphotransferase activity but contain nearly normal levels of lysosomal enzymes (*7*, *8*). It was not demonstrated, however, whether an alternative pathway in these cells functions only in the absence of Man 6-P–dependent transport due to the lack of phosphotransferase. In addition, there is growing evidence for a Man 6-P–independent pathway in several cell types (*11–15*). All these studies indicate the involvement of a transient membrane association of a precursor independent of Man 6-P, although it is not clear whether the Man 6-P–dependent and independent pathways operate as distinct sorting steps or whether the pathways comprise a sequential process.

In the present paper, we ask whether hepatocytes isolated from normal rat liver contain a Man 6-P–dependent pathway that contributes to the targeting of cathepsin B, a major lysosomal cysteine protease, to lysosomes, because despite the presence of Man 6-P generating enzymes and MPRs, hepatocytes are one of the cell types that utilize a Man 6-P–independent pathway in the lysosomal targeting of lysosomal enzymes as described for ICD. We show here that cathepsin B acquires the Man 6-P signal only on an oligosaccharide chain in the propeptide region, and that nearly all phosphorylated procathepsin B is secreted instead of being delivered to lysosomes. Therefore, the results indicate that there is no coupling between the acquisition of a Man 6-P signal and lysosomal targeting of cathepsin B. Moreover, about 50% of the newly synthesized cathepsin B destined for lysosomes appears to be targeted by a Man 6-P–independent mechanism(s).

MATERIALS AND METHODS

Materials—Culture media and fetal calf serum were purchased from GIBCO BRL (Grand Island, NY). [^{32}P]Phosphate and EXPRE^{35}S^{35}S labeling mix were from DuPont-NEN (Boston, MA). Brefeldin A (BFA) was purchased from Epicentre Technologies (Madison, WI). β-Endo-*N*-acetylglucosaminidase H (Endo H) was from Seikagaku (Tokyo). Peptide-*N*-glycosidase F (PNGase F) and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, IN). The anti–cathepsin B antisera used in the experiments were described earlier (*16*). Rat liver cathepsin D was purified as described by Yamamoto *et al.* (*17*).

Metabolic Labeling and Immunoprecipitation—Hepatocytes isolated from male Wistar rats (200 g) by collagenase-perfusion (*18*) were diluted with DMEM containing 10% fetal calf serum, seeded on collagen-coated tissue culture dishes (35 mm in diameter), and incubated in humidified air containing 5% CO_2 at 37°C for 24 h. The cells were preincubated for 1 h with phosphate-free DMEM containing 5% fetal calf serum, then labeled for 3 h (Figs. 1 and 2) or 2 h (Fig. 6) with 250 μCi of [^{32}P]phosphate. Chase was initiated by the addition of sodium phosphate buffer, pH 7.4, to 10 mM. For [^{35}S]methionine/cysteine labeling, the cells were preincubated for 1 h with methionine- and cysteine-free DMEM containing 5% fetal calf serum, then labeled for 3 h (Figs. 1 and 2) or 2 h (Fig. 6) with 100 μCi/ml of EXPRE^{35}S^{35}S labeling mix. Chase was initiated by adding unlabeled methionine to 10 mM. For BFA treatment, the preincubation and labeling media were supplemented with 10 μg/ml BFA. At the end of the pulse or chase, the media were collected and the cells were harvested by the addition of 0.5 ml of lysis buffer [50 mM sodium phosphate buffer, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% bovine serum albumin (w/v), 5 mM β-glycerophosphate, 50 mM NaF, 0.1 mM *o*-vanadate, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, and protease inhibitor mixture containing 2 μg/ml antipain, chymostatin, leupeptin, and pepstatin A], and immunoprecipitated as described (*18*). The precipitates were washed as described (*18*), and analyzed by SDS-PAGE according to Laemmli (*19*) using 10% acrylamide. Labeled polypeptides were visualized and quantitated with a Fuji BAS 2000 Imaging Analyzer.

Glycosidase Digestion—After immunoprecipitation, immune complexes were dissociated by heating for 3 min at 95°C in 40 μl of 50 mM sodium citrate buffer, pH 5.5, containing 0.5% SDS and 0.1 M β-mercaptoethanol. After centrifugation, the supernatant was collected, and 40 μl of 50 mM sodium citrate buffer, pH 5.5, containing 2% Nonidet P-40 and 2 mM PMSF was added. The sample was divided into equal aliquots and incubated in the presence or absence of 5 milliunits of Endo H for 16 h at 37°C. For PNGase F digestion, immunoprecipitates were dissociated by heating for 3 min at 95°C in 40 μl of 0.1 M sodium phosphate buffer, pH 8.6, containing 0.5% SDS and 0.1 M β-mercaptoethanol. After centrifugation, the supernatant was collected, and 40 μl of 0.1 M sodium phosphate buffer, pH 8.6, containing 2% Nonidet P-40, 5 mM EDTA, and 2 mM PMSF was added. The sample was divided into equal aliquots and incubated in the presence or absence of 10 units/ml of PNGase F for 24 h at 30°C.

Alkaline Phosphatase Digestion—Treatment with alkaline phosphatase was done by the procedure described by Isidoro *et al.* (*20*). Immunoprecipitates were solubilized by heating for 5 min at 95°C in 40 μl of 0.6% SDS and 60 mM Tris/HCl, pH 8.8. After centrifugation, the supernatant was collected and diluted with 2 volumes of distilled water. The sample was divided into equal aliquots and each aliquot was incubated in the presence or absence of 12.5 units of calf intestine alkaline phosphatase for 16 h at 37°C.

Processing of Procathepsin B with Cathepsin D—Immunoprecipitates were incubated for 1 h at 4°C in 40 μl of 0.1 M sodium formate buffer, pH 3.0. After centrifugation, the supernatant was collected, and the samples were incubated with 0.5 μg of cathepsin D for 1 h at 30°C in the presence of 2 μg/ml of E64.

CI-MPR Affinity Chromatography—The cells were labeled with [^{32}P]phosphate for 3 h and chased for 6 h. The secretions were collected and precipitated with ammonium sulfate (50% w/v). The precipitates were dialyzed against CI-MPR binding buffer (50 mM imidazol-HCl, pH 6.5, 5 mM Na β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.05% Triton X-100) and applied to a CI-

MPR column. After washing the column with binding buffer and 5 mM glucose 6-phosphate in binding buffer, the materials were eluted with 10 mM mannose 6-phosphate in binding buffer and cathepsin B was immunoprecipitated as described above.

Membrane Association of Procathepsin B—The cells were labeled with [^{35}S]methionine/cysteine for 3 h, washed extensively with PBS, and harvested in 10 mM sodium phosphate buffer, pH 7.4, containing proteinase inhibitors, 2 mM EDTA, and 5 mM mannose 6-phosphate. The suspensions were frozen and thawed (repeated three times) and centrifuged at 105,000 ×*g* for 1 h. The supernatant was retained for immunoprecipitation, and the resulting pellet was extracted with 100 mM Na_2CO_3 containing proteinase inhibitors. The suspension was re-centrifuged, and the supernatant was adjusted to pH 7.4 and retained for immunoprecipitation. The pellet was lysed with 10 mM sodium phosphate buffer, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, and protease inhibitors. Cathepsin B was immunoprecipitated from each of the three extracts.

RESULTS

Phosphorylation of Cathepsin B—We first examined whether cathepsin B is phosphorylated on its *N*-linked oligosaccharide side chains. Primary cultured hepatocytes were labeled with [^{32}P]phosphate for 3 h, and cathepsin B was immunoprecipitated from the cells. The immunoprecipitates were digested with or without Endo H. As shown in Fig. 1, ^{32}P-labeled cathepsin B was detected as a single band with an apparent molecular mass of 39-kDa. Upon treatment with Endo H, ^{32}P incorporated into cathepsin B disappeared, indicating that the phosphorylation of cathepsin B occurs only on its high mannose type oligosaccharide side chains. This 39-kDa polypeptide seems most likely to be a precursor form of cathepsin B, since the same size cathepsin B, which is sensitive to Endo H, was obtained when the cells were labeled with [^{35}S]methionine/cysteine for 30 min (Fig. 3), and had the same molecular mass as cathepsin B purified from rat liver microsome fractions (*16*).

We then asked whether phosphorylated oligosaccharide chains in cathepsin B are processed to a phosphomonoester form, since the efficiency of segregation and targeting of soluble lysosomal enzymes depends on the formation of Man 6-P monoester, which has a high affinity for Man 6-P receptors (*1, 2*). At the end of the pulse (3 h) and chase periods (6 h), cathepsin B was immunoprecipitated from the cell lysates and media. The immunoprecipitates were further digested with or without alkaline phosphatase in order to analyze the acquisition of Man 6-P monoester. After labeling for 3 h, approximately 60% of the [^{32}P]phosphate incorporated into intracellular procathepsin B was released by alkaline phosphatase treatment (Fig. 2A), a result that was not affected by a prolonged incubation period or increased enzyme concentration (data not shown). Interestingly, after a 6 h chase, phosphorylated cathepsin B was no longer detected. In contrast, large amounts of phosphorylated procathepsin B were secreted into the medium, even when the cells were labeled for 3 h. After the 6 h chase, phosphorylated cathepsin B was detectable only in the medium. During the pulse/chase experiments, more than 95% of the ^{32}P in secreted procathepsin B proved to be sensitive to alkaline phosphatase. These results indicate that despite the acquisition of Man 6-P residues, a correct targeting signal for the MPR-dependent lysosomal delivery system (*1, 2*), part of the phosphorylated procathepsin B was secreted into the extracellular medium without being transported to lysosomes.

On the other hand, when the cells were labeled for 3 h with [^{35}S]methionine/cysteine, a 29-kDa polypeptide corresponding to a single chain form of mature type cathepsin B (*16*), as well as a small amount of 39-kDa procathepsin B, was already visible as a major polypeptide in the cells (Fig. 2B). Following a chase period of 6 h, an additional 24-kDa two chain form polypeptide also appeared in the cells. As observed with ^{32}P-labeling (Fig. 2A), secretion of procathepsin B was already apparent during a labeling period of 3 h, and, eventually, ~50% of the cathepsin B was secreted as a proform into the medium after chase for up to 6 h, indicating that about half the amount of cathepsin B in rat hepatocytes is constitutively secreted without undergoing proteolytic processing and segregation from secretory proteins.

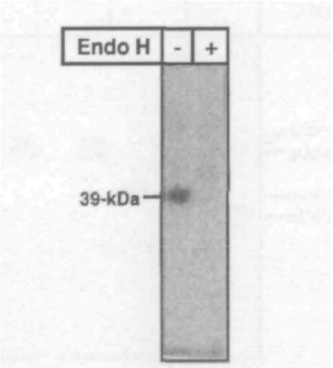

Fig. 1 **Phosphorylation of cathepsin B in rat hepatocytes.** Primary cultured rat hepatocytes were labeled with [^{32}P]phosphate for 3 h, and cathepsin B was immunoprecipitated from the cells. Immunoprecipitates were incubated in the presence (+) or absence (−) of Endo H and analyzed by SDS-PAGE. The apparent molecular masses (kDa) are indicated

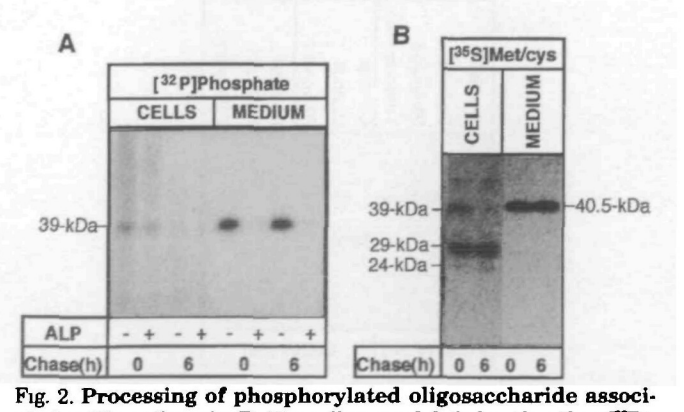

Fig. 2. **Processing of phosphorylated oligosaccharide associated with cathepsin B.** *The cells were labeled with either* [^{32}P]-phosphate (A) or [^{35}S]methionine/cysteine (B) for 3 h and chased for 0 or 6 h, as indicated. Cathepsin B was immunoprecipitated from the cells and media. In the case of ^{32}P-labeled cells immunoprecipitates were incubated in the presence (+) or absence (−) of alkaline phosphatase.

The ^{35}S-labeled procathepsin B secreted into the medium exhibited slower electrophoretic mobility than that in the cells (Fig 2B), an event related to the processing of the high mannose type oligosaccharide chain to the complex type chain (Fig. 3). Upon digestion with Endo H, cell-associated 39-kDa procathepsin B shifted to a 36-kDa form that had the same electrophoretic mobility as that prepared by either PNGase F digestion of procathepsin B or by tunicamycin-treated cells. In contrast, Endo H digestion of the secreted form with an apparent molecular mass of 40.5-kDa demonstrated about 80% of the secreted form to be Endo H-resistant; about 20% was partially Endo H sensitive and underwent an apparent molecular mass reduction from 40.5- to 38.5-kDa. PNGase F digestion of the secreted form also resulted in a single band with an apparent molecular mass of 36-kDa, the same molecular mass as that immunoprecipitated from cells treated with tunicamycin. Thus, the partially Endo H-sensitive form comprises a mixture of one high mannose type and one complex type *N*-linked oligosaccharide chain. This partially Endo H sensitive form probably migrates in the lower part of the 40.5-kDa band, because no significant difference in molecular masses between ^{32}P-labeled intracellular and extracellular procathepsin B was observed (Fig. 2A).

Phosphorylation Site of Procathepsin B—It is noteworthy that neither the 29-kDa single chain form nor the 24-kDa two chain form was detected in the ^{32}P-pulse/chase experiments (Fig. 2, A and B). Even when cells labeled for 3 h with [^{32}P]phosphate were chased for various intervals (0.5, 1, and 2 h), the phosphorylated single chain and two chain forms were not detected (data not shown). The failure to observe any phosphorylation of the two forms could not be accounted for by rapid dephosphorylation.

It is of interest to note that cathepsin B contains two positive sites for *N*-linked glycosylation, at Asn-21 and Asn-272 (*21*), and that both sites seem likely to be utilized. The site at Asn-21 is included in the prosequence and is concomitantly removed during processing of the propeptide. We considered an alternative explanation for our results; the detectable phosphorylated cathepsin B molecule was perhaps only a proform and only an oligosaccharide chain on Asn-21 present in the propeptide might undergo phosphorylation. We therefore proceeded to determine the site of the phosphorylated oligosaccharide chain.

We have reported that procathepsin B can be selectively cleaved at a position between Gly-48 and Pro-49 in its propeptide by *in vitro* incubation with cathepsin D at pH 3.0, yielding an intermediate form with an apparent molecular mass of 31-kDa, ~2-kDa larger than the 29-kDa single chain form present in lysosomes (*16*). Since processing of the propeptide by cathepsin D is accompanied by the removal of an oligosaccharide chain present at Asn-21, we took advantage of this to identify the site of phosphorylation. Figure 4 shows the results of typical experiments in which the cells were labeled for 3 h with either [^{32}P]phosphate or [^{35}S]methionine/cysteine, and immunoprecipitates from cell lysates and media were treated with or without cathepsin D. Upon incubation with cathepsin D, ^{35}S-labeled intracellular procathepsin B with an apparent molecular mass of 39-kDa underwent a decrease in molecular mass to 31-kDa. This difference in size seems likely to correspond to the molecular mass of the 48 amino acid residues plus one oligosaccharide chain that are expected to be cleaved by cathepsin D. Likewise, digestion of extracellular 40.5-kDa procathepsin B by cathepsin D resulted in a decrease in the molecular mass to 31.5-kDa, which is slightly larger than that of intracellular procathepsin B. This can be probably accounted for by processing to the complex type of oligosaccharide in the mature region, as described below. In contrast to the situation with [^{35}S]methionine/cysteine, no ^{32}P-labeled cathepsin B band was detected when ^{32}P-labeled procathepsin B immunoprecipitated from either cell lysates or medium was incubated with cathepsin D. These results show that the phosphorylation of cathepsin B occurs only on the oligosaccharide chain of Asn-21 present in the pro-

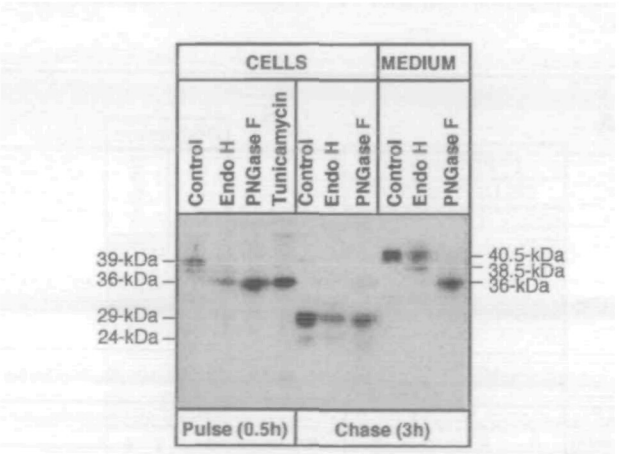

Fig. 3. **Processing of *N*-linked oligosaccharide chains of cathepsin B.** The cells were labeled for 30 min and chased for 3 h, as indicated. For tunicamycin treatment, the cells were preincubated for 5 h with 5 μg/ml of tunicamycin, and labeled for 30 min in the presence of this drug. Cathepsin B was immunoprecipitated from the cells and media, and the precipitates were subjected to SDS-PAGE, directly or after glycosidase digestion, as indicated.

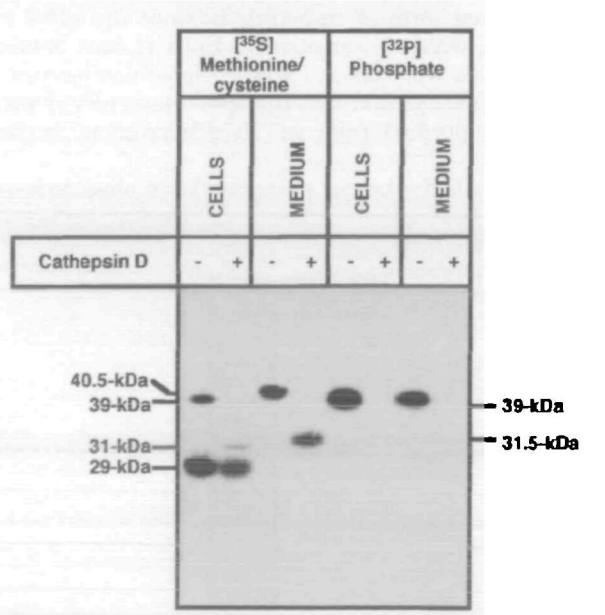

Fig. 4. **Phosphorylation site of procathepsin B.** The cells were labeled with either [^{32}P]phosphate (A) or [^{35}S]methionine/cysteine (B) for 3 h and chased for 0 or 6 h, as indicated. Cathepsin B was immunoprecipitated from the cells and media and incubated with 0.5 μg of purified rat liver cathepsin D in the presence of E64 at pH 3 and 30°C for 1 h

peptide. Moreover, oligosaccharides on Asn-272 in the mature portion of the enzyme were processed to the complex type, because the 31.5-kDa form produced by cathepsin D was resistant to Endo H but reduced 2.5-kDa by treatment with PNGase F (data not shown).

Effect of NH_4Cl on the Secretion of Phosphorylated Procathepsin B—The results in Fig. 4 showing that the phosphorylation of cathepsin B occurs on an oligosaccharide chain present in the propeptide further imply that it is not possible to obtain direct evidence as to whether phosphorylated cathepsin B itself is indeed targeted to the lysosomes, because of the cleavage of the propeptide containing the phosphorylated oligosaccharide chain en route to or immediately after arrival at lysosomes. Therefore, to facilitate analysis of lysosomally targeted phosphorylated cathepsin B molecules, we examined the effect of NH_4Cl on the secretion of phosphorylated procathepsin B. NH_4Cl causes an increase in the pH of the endosomal/lysosomal system (*22*) and prevents intracellular proteolytic processing and segregation of newly synthesized lysosomal enzymes from the secretory route leading to enhancement of secretion of Man 6-P–bearing lysosomal enzyme precursors (*23*, *24*).

First, we examined the effect of NH_4Cl on the secretion of newly synthesized cathepsin B by pulse/chase experiments with [^{35}S]methionine/cysteine. As shown in Fig. 5A, during 3-h labeling in the presence of 10 mM NH_4Cl, the extent of secretion of procathepsin B (47% of the total) was close to that in the control cells (42% of the total). However, in the treated cells, most of the cathepsin B remaining intracellularly was in a precursor form, while a small amount of the 32-kDa form, which probably represents the intermediate form, was being processed. After a 6 h chase in the pres-

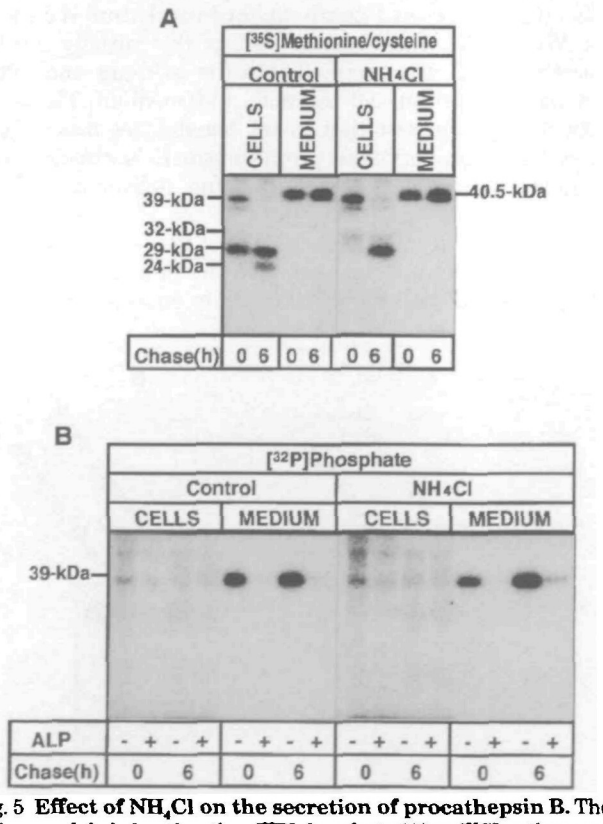

Fig. 5 **Effect of NH_4Cl on the secretion of procathepsin B.** The cells were labeled with either [^{32}P]phosphate (A) or [^{35}S]methionine/cysteine (B) for 3 h and chased for 0 or 6 h, as indicated, in the presence of 10 mM NH_4Cl. Cathepsin B was immunoprecipitated from the cells and media and analyzed by SDS-PAGE. In the case of ^{32}P-labeled cells, immunoprecipitates were incubated in the presence (+) or absence (−) of alkaline phosphatase

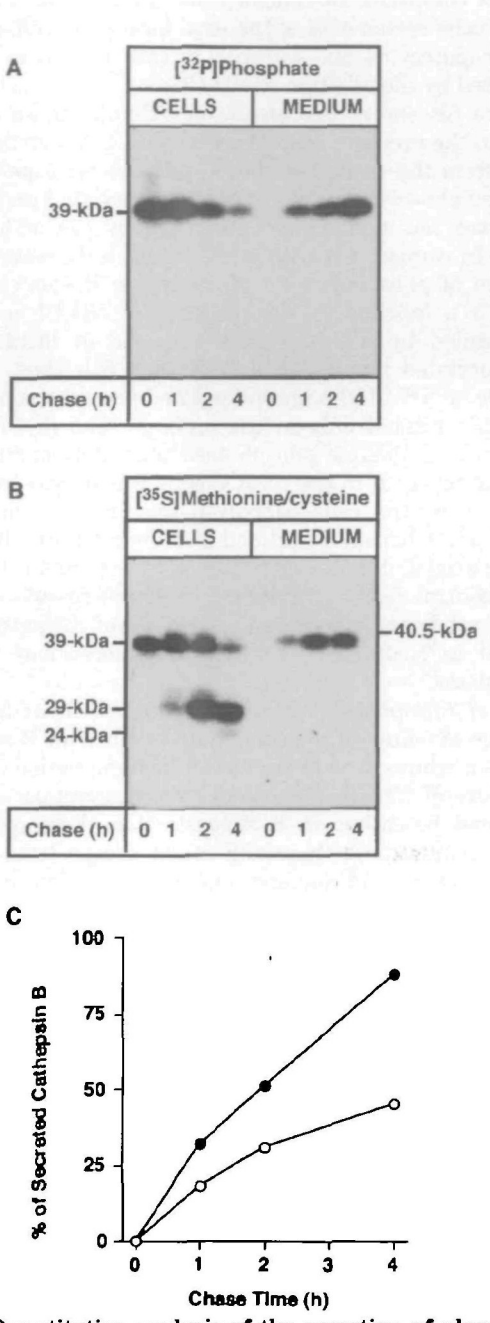

Fig 6 **Quantitative analysis of the secretion of phosphorylated cathepsin B.** Cells were incubated for 1 h in the presence of 10 μg/ml of BFA, labeled with either [^{32}P]phosphate (A) or [^{35}S]methionine/cysteine (B) for 2 h in the presence of 10 μg/ml of BFA, and chased for the indicated time in the absence of BFA. Cathepsin B was immunoprecipitated from the cells and media and analyzed by SDS-PAGE. (C) The fluorograms shown in A and B were quantitated by scanning densitometry Values are expressed as percentages of the secreted cathepsin B detected at a given level relative to the intracellular procathepsin B detected at 0 h of chase The closed and open circles represent analyses of ^{32}P- and ^{35}S-labeled cathepsin B secreted, respectively. The results are the means of three separate experiments.

ence of NH_4Cl, the majority of the procathepsin B that remained intracellularly after pulse-labeling was processed to the 29-kDa single chain form, but not to the 24-kDa two chain form. Although the amount of procathepsin B secreted increased slightly, the extent was only a ~1.2-fold increase compared to control cells (57% of the total for treated cells *versus* 47% of the total for control cells). Thus, in rat hepatocytes, the secretion of cathepsin B is largely unaffected by the addition of NH_4Cl.

Figure 5B shows the results of ^{32}P-pulse/chase experiments in the presence or absence of NH_4Cl. As can be anticipated from the results of the ^{35}S-pulse/chase experiments described above, the secretion of phosphorylated procathepsin B was not significantly enhanced by the addition of NH_4Cl. In comparison with control cells, a decrease in the secretion of phosphorylated procathepsin B was observed during 3 h labeling in the presence of NH_4Cl, and was accompanied by a concomitant increase in intracellular phosphorylated procathepsin B. After a 6 h chase in the presence of NH_4Cl, the phosphorylated procathepsin B was eventually located only in the medium. The distribution, intensity and alkaline phosphatase sensitivity of ^{32}P-labeled procathepsin B in the treated cells was mostly identical to that in control cells. Moreover, the $^{32}P/^{35}S$ ratio of the labeled procathepsin B secreted into the medium after a 6-h chase was slightly lower in the NH_4Cl-treated cells than in the control cells (1.2 *versus* 1.4). These results suggest that in rat hepatocytes most, if not all, of cathepsin B is targeted to lysosomes by Man 6-P–independent sorting mechanisms.

Fate of Phosphorylated Procathepsin B—In addition to the large amounts of phosphorylated cathepsin B secreted into the medium even during a 3-h labeling period (Fig. 2), the failure of NH_4Cl to cause enhanced secretion of phosphorylated procathepsin B suggests that the majority of phosphorylated procathepsin B might escape transport to the lysosomes, and instead undergo secretion into the medium. Alternatively, the phosphorylated procathepsin B found in the medium might be a minor fraction, with most of the phosphorylated procathepsin B having been delivered to the lysosomes, but undetectable due to cleavage of the propeptide, including the phosphorylated oligosaccharide chain.

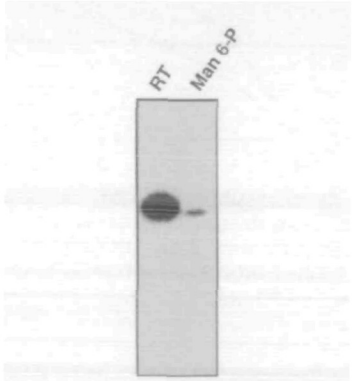

Fig. 7. **CI-MPR affinity chromatography.** The cells were labeled with [^{32}P]phosphate for 3 h and chased for 6 h. Media were collected and applied to a CI-MPR column as described under "MATERIALS AND METHODS." Cathepsin B was immunoprecipitated from the run through (RT) and 10 mM Man 6-P eluted (Man 6-P) fractions and analyzed.

To address these questions, we attempted to trace the fate of phosphorylated procathepsin B retained intracellularly. For this purpose, we used a fungal metabolite, BFA, to accumulate phosphorylated procathepsin B within the cells. BFA blocks secretion from the ER/Golgi system to allow redistribution of Golgi proteins into the ER, and, consequently, newly synthesized proteins accumulate within the mixed ER/Golgi system and are processed by the redistributed Golgi enzymes (*25–28*). The effect of BFA is rapidly and completely reversed upon removal of the drug. Indeed, BFA blocks the transport of newly synthesized lysosomal enzymes to lysosomes (*29*) but does not affect the incorporation of [^{32}P]phosphate into cathepsin D'oligosaccharide side chains (*30*).

Figure 6, A and B, shows the results of an experiment in which the cells were labeled with either [^{32}P]phosphate or [^{35}S]methionine/cysteine, respectively, for 2 h in the presence of BFA (10 μg/ml) to cause phosphorylated procathepsin B to be retained intracellularly, and then chased for up to 4 h in the absence of BFA to restore normal transport. These experimental conditions did not significantly affect the biosynthesis and intracellular transport of cathepsin B, as judged from the results obtained in ^{35}S-labeling experiments (Fig. 6B). As is evident in comparison with control cells (Fig. 2A), the addition of BFA to the cells during pulse-labeling resulted in a remarkable intracellular accumulation of ^{32}P-labeled procathepsin B (Fig. 6A). When the cells were subsequently chased with BFA-free medium, ^{32}P-labeled procathepsin B progressively disappeared from the cells and was secreted nearly quantitatively into the medium. With a 4 h chase, about 90% of the initially labeled procathepsin B was secreted into the medium and ~90% was recovered from cell extracts and medium. These results strongly suggest that in rat hepatocytes most, if not all, of the phosphorylated procathepsin B is directed to a secretory pathway rather than being delivered to lysosomes.

CI-MPR Affinity Chromatography of Phosphorylated Procathepsin B—We further examined the binding of secreted procathepsin B with CI-MPR. Medium obtained from cells

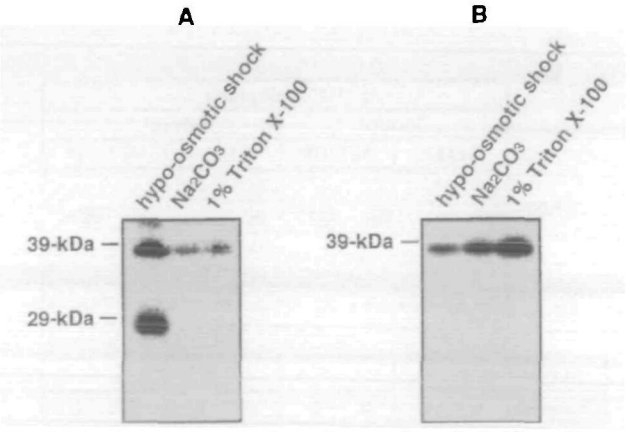

Fig. 8. **Membrane association of procathepsin B.** The cells were labeled with [^{35}S]methionine/cysteine for 3 h in the absence (A) or presence of 10 μg/ml BFA (B) and extracted sequentially by hypo-osmotic shock in the presence of 10 mM Man 6-P, 100 mM Na_2CO_3, and 1% Triton X-100 as described under "MATERIALS AND METHODS." Cathepsin B was immunoprecipitated from each extract and analyzed by SDS-PAGE.

subjected to 3-h labeling with a 6-h chase were applied to a CI-MPR column and eluted with 10 mM Man 6-P. As shown in Fig. 7, more than 90% of the ^{32}P-labeled procathepsin B was immunoprecipitated from the flow through and washing fractions, and less than 10% of the procathepsin B was eluted by 10 mM Man 6-P, indicating weak affinity of the phosphorylated procathepsin B for CI-MPR.

Membrane Association of Procathepsin B—Various lines of evidence have revealed that several lysosomal enzymes are transported to the endosomes and/or lysosomes in membrane-associated forms and a Man 6-P–independent manner (*11–15*). In order to determine whether newly synthesized cathepsin B is associated with the membrane in a Man 6-P–independent manner, the cells were labeled with [^{35}S]methionine/cysteine for 3 h and sequentially extracted by hypo-osmotic shock, Na_2CO_3 and Triton X-100. As shown in Fig. 8A, all mature cathepsin B was recovered in the hypo-osmotic shock fraction. About 60 of the procathepsin B was extracted by hypo-osmotic shock and 20% by Na_2CO_3 treatment, but 20% was released by 1% Triton X-100. Under the same condition, albumin and LGP107, an integral lysosomal membrane protein, appeared exclusively in the hypo-osmotic shock and Triton X-100 extract fractions, respectively (data not shown). To examine further whether the membrane-association of procathepsin B occurs in the early stages of the secretory route, cells labeled with [^{35}S]-methionine/cysteine for 3 h in the presence of BFA were subfractionated as described above. As shown in Fig. 8B, the amount of membrane-associated procathepsin B increased to about 60% of the total in BFA treated cells. Thus, part of the newly synthesized procathepsin B can associate with intracellular membranes in an early secretory pathway in a Man 6-P–independent manner.

DISCUSSION

The results presented here demonstrate that cathepsin B synthesized by rat hepatocytes acquires Man 6-P residues only on an oligosaccharide in the propeptide region. Despite the acquisition of the Man 6-P recognition marker, nearly all of the phosphorylated cathepsin B is secreted without being delivered to lysosomes. Our results show that the intracellular transport of phosphorylated cathepsin B to lysosomes apparently does not involve a Man 6-P–dependent mechanism.

It is generally accepted that the phosphorylation of oligosaccharide chains on lysosomal enzymes occurs on high-mannose type oligosaccharides, and that after phosphorylation, it is no longer processed to the complex type during passage through the Golgi (*1*, *2*). Consistent with this notion, we also observed that cathepsin B is phosphorylated on Endo H–sensitive high-mannose type oligosaccharide chains. From the results of both Endo H– and PNGase F–digestions (Fig. 3), and *in vitro* processing experiments by cathepsin D (Fig. 4), it is apparent that the phosphorylation of cathepsin B occurs only on the oligosaccharide in the propeptide region while the oligosaccharide in the mature region is processed to a complex type. We could not determine, however, whether an oligosaccharide chain present in the mature region that is destined to be delivered to lysosomes is a high mannose type or complex type, because cathepsin B acquires both Endo H and PNGase F resistance immediately after proteolytic processing. On the other hand, it has been reported that in human fibroblasts both the 44.5-kDa precursor and 33-kDa forms of cathepsin B are phosphorylated (*31*). Although the molecular masses of cathepsin B in human fibroblasts are different from those in rat hepatocytes, the 33-kDa form may correspond to the 29-kDa mature form in rat hepatocytes, since it is the main form present in human fibroblasts as determined by immunoblot analysis. At present, it is not possible to explain the difference in the acquisition of Man 6-P in the mature form. Studies of the oligosaccharide structure of cathepsin B have revealed, however, that the purified mature forms from rat liver (*32*) and porcine spleen (*33*) have unique oligosaccharide structures, containing only 4- and 5-sugar, respectively. These results support our results that there is no difference in the electrophoretic mobility of the mature form after glycosidase digestion.

Although the majority of lysosomal enzymes contain two or more *N*-linked oligosaccharides, site-specific phosphorylation has been reported for several lysosomal enzymes, including the α- (*34*) and β-subunits (*35*) of human β-hexosaminidase, human α-glucosidase (*36*), human β-glucuronidase (*37*), human arylsulfatase A (*38*), and β-D-galactosidase and *N*-acetyl-α-neuraminidase (*39*). For example, arylsulfatase A has three potential *N*-glycosylation sites on Asn residues 158, 184, and 350, all of which are utilized. A mutant arylsulfatase A containing only an oligosaccharide at Asn-184 produced by site-directed mutagenesis was phosphorylated (*38*), but the phosphorylation site of the wild type enzyme expressed in BHK cells was found to be re-stricted to oligosaccharides at Asn-158 and Asn-350, based on analyses of tryptic fragments (*40*). It was postulated that *in vivo*, the phosphorylation of one oligosaccharide could alter the phosphorylation of another oligosaccharide. It is conceivable, therefore, that phosphorylation of the oligosaccharide in the propeptide region of cathepsin B renders the oligosaccharide in the mature region inaccessible to phosphotransferase.

The existence of a single phosphorylated oligosaccharide might explain why, despite the acquisition of Man 6-P residues, most of the phosphorylated procathepsin B is quantitatively secreted into the medium without being dephosphorylated in the cells. In general, high affinity binding of lysosomal enzymes to MPRs is explained by the 2-site model (*41*). In this case, Man 6-P residues on separate oligosaccharides can interact with MPRs of higher affinity than the two Man 6-P residues on the same oligosaccharides. For example, mouse cathepsin L, which is secreted selectively from NIH3T3 cells, has a low affinity for CI-MPR, and contains a single oligosaccharide with two Man 6-P residues (*42*). In contrast, cathepsin L made by CHO cells has a higher affinity for the receptor than does the mouse enzyme, and contains two oligosaccharides with two Man 6-P residues. The low affinity of mouse cathepsin L for the receptor is attributed to the existence of a single oligosaccharide. Indeed, we observed that most of the phosphorylated procathepsin B did not bind to CI-MPR (Fig. 7). Alternatively, the Man 6-P residue attached to the oligosaccharide chain on the propeptide may become inaccessible for binding to MPRs, probably due to masking of the Man 6-P residue by ionic interaction with positively charged amino acids in the protein backbone, as suggested previously by Lazzarino and Gabel (*43*). We cannot rule out the possibility, however, that the secretion of phosphorylated

procathepsin B might be mediated by CD-MPR, since it appears to function both in the secretion of newly synthesized lysosomal enzymes and in their intracellular targeting to lysosomes (*44*). Recent studies have revealed that both MPRs have largely overlapping but apparently distinct affinities for lysosomal enzymes (*3–5*).

Interestingly, our results indicate that phosphorylated cathepsin B is a minor fraction. While nearly all of the phosphorylated procathepsin B is secreted into the medium, about 50% of the ^{35}S-labeled cathepsin B is secreted into the medium, of which only about 20% is partially sensitive to Endo H. Since Endo H treatment resulted in a complete disappearance of ^{32}P-labeled cathepsin B, when roughly estimated, about 10% of the newly synthesized cathepsin B appears to be phosphorylated during biosynthesis. Although the reason for this lower level of phosphorylation is not clear, procathepsin B may be a poor substrate for phosphotransferase. It is interesting to note that a similar low level of phosphorylation has been reported for bovine and mouse DNase I, a secretory glycoprotein of the pancreas and the salivary gland (*45, 46*). In these cases, the poor phosphorylation is explained by either the absence of amino acids that are essential for efficient phosphorylation or the presence of amino acids that impair phosphorylation. Anyway, the low level of phosphorylation, together with the absence of an effect of NH_4Cl on increased secretion of phosphorylated procathepsin B, and the secretion of nearly all the phosphorylated procathepsin B, do indicate that in rat hepatocytes, the Man 6-P–dependent pathway probably is not a major mechanism that contributes to the targeting of newly synthesized cathepsin B to lysosomes.

In contrast to the results that most of phosphorylated procathepsin B is secreted into the medium rather than being dephosphorylated within the cells, ^{35}S-pulse/chase experiments revealed that 50% of the newly synthesized cathepsin B was processed to the mature forms, an indication of transport to lysosomes. These results, clearly, indicate that 50% of the newly synthesized cathepsin B is delivered to lysosomes without being phosphorylated, thereby demonstrating the targeting to lysosomes *via* a Man 6-P–independent pathway. In contrast, human fibroblasts target newly synthesized cathepsin B to lysosomes by a Man 6-P dependent pathway (*31*). This comes from the results that NH_4Cl treatment leads to the enhanced secretion of procathepsin B into the culture medium, and >95% of the newly synthesized procathepsin B is secreted in ICD fibroblasts. It should be pointed out, however, that in cytolytic ICD lymphocytes, in which the Man 6-P–dependent pathway is inoperable due to a deficient phosphotransferase, cathepsin B is localized in lytic granules that perform both lysosomal and secretory roles (*47*). Thus a different targeting pathway of cathepsin B in different cell lines is in accord with the notion that, in contrast to fibroblasts, some tissues, including liver and some cell types, utilize an alternative pathway for the targeting of lysosomal enzymes to lysosomes (*5, 6*). It has been shown that in thymocytes and hepatocytes from mice deficient in CD-MPR, CI-MPR, and insulin-like growth factor II, cathepsin D is targeted to lysosomes in a Man 6-P–independent manner (*48*). Although newly synthesized cathepsin D is transported along an intracellular route in thymocytes, hepatocytes target newly synthesized cathepsin D *via* a secretion–recapture and/or a direct cell-to-cell contact. Secretion-recapture mechanisms *via* mannose- and/or galactose-specific receptors have been shown to be involved in the correction of the missorting of lysosomal enzymes in CD-MPR–deficient mice (*49*), indicating a Man 6-P–independent pathway.

Another possible Man 6-P–independent targeting mechanism involves the transient interaction with intracellular membranes. Man 6-P–independent membrane association has been demonstrated for procathepsin D, β-glucocerebrosidase, and prosaposin in HepG2 cells (*13*), procathepsin D in macrophages (*11*) and in human breast cancer cells (*15*), procathepsins D and L in mouse fibroblasts (*14*), and procathepsin C in Morris hepatoma 7777 cells (*12*). We found that part of the newly synthesized procathepsin B was associated with membranes in a Man 6-P–independent manner, but the single chain form was in a soluble form, indicating proform-specific membrane association. This does not necessarily imply, however, that the propeptide of cathepsin B is responsible for Man 6-P–independent membrane association, since the membrane association of cathepsin D in HepG2 cells takes place independent of the removal of the propeptide (*50*). Further study is required to define the role of the propeptide in the Man 6-P–independent membrane association. Our results further indicate that the membrane-association of procathepsin B occurs already at an early stage of the biosynthetic pathway (Fig. 8B). This leads us to speculate that the membrane-association of procathepsin B involved in Man 6-P–independent lysosomal targeting does not require an acidic environment. This speculation is also supported by the fact that NH_4Cl does not prevent the proteolytic processing of procathepsin B to the single chain form, as well as the failure of the enhanced secretion (Fig. 4). However, since NH_4Cl resulted in the retardation of the proteolytic processing of procathepsin B, we cannot rule out the possibility that a pH-dependent membrane-association is also involved in the effective Man 6-P–independent lysosomal targeting of procathepsin B. It is conceivable, therefore, that the Man 6-P–independent membrane association of procathepsin B is already initiated in the ER and/or Golgi complex and continues in endosomes. Similar results have been reported for procathepsin D in HepG2 cells (*50*).

On the other hand, Authier *et al.* (*51*) conducted immunoblot analysis Man 6-P–independent membrane association of both the pro- and mature forms of cathepsin B using endosomal membranes isolated from rat liver. In this case, both the single and two chain forms were tightly associated with the endosomal membranes. However, our results indicated that the processing to the two chain form occurs after 6 h chase, and that at that time the two chain form is recovered only in the lysosomal fraction, although the single chain form appears in both the lysosomal and in the light density fraction containing endosomes, but in a soluble form (data not shown). The association of the mature form with endosomal membranes, therefore, may reflect a resident rather than a transient passenger during the biosynthetic pathway. We assume that most, if not all, of the procathepsin B bound to the membranes, is delivered to endosomes, where it is processed to the soluble single chain form either in endosomes or in lysosomes, or both. Sequential processing to the two chain form on the other hand occurs in lysosomes.

In this study, we have shown that the Man 6-P–dependent pathway does not contribute to lysosomal targeting of

cathepsin B in rat hepatocytes, and that hepatocytes are likely to possess an alternative targeting system for introducing cathepsin B into lysosomes. It is possible that the Man 6-P–independent membrane association contributes targeting of cathepsin B to lysosomes in rat hepatocytes.

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