Expression of Human Cathepsin D in *Xenopus* Oocytes: Phosphorylation and Intracellular Targeting

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Abstract. We have obtained expression of a cDNA clone for human cathepsin D in *Xenopus laevis* oocytes. Biosynthetic studies with [^{35}S]methionine labeling demonstrated that most of the cathepsin D remained intracellular and underwent proteolytic cleavage, converting a precursor of M_r 47,000 D to a mature form of M_r 39,000 D with processing intermediates of M_r 43,000–41,000 D. >90% of the cathepsin D synthesized by oocytes bound to a mannose 6-phosphate (Man-6-P) receptor affinity column, indicating the presence of phosphomannosyl residues. An analysis of [2-^{3}H]mannose-labeled oligosaccharides directly demonstrated phosphomannosyl residues on cathepsin D. Sucrose-gradient fractionation, performed to define the membranous compartments that cathepsin D traversed during its biosynthesis, demonstrated that cathepsin D is targeted to a subpopulation of yolk platelets, the oocyte equivalent of a lysosome. *Xenopus* oocytes were able to endocytose lysosomal enzymes from the medium and this uptake was inhibited by Man-6-P, thus demonstrating the presence of Man-6-P receptors in these cells. Therefore, the entire Man-6-P dependent pathway for targeting of lysosomal enzymes is present in the oocytes. *Xenopus* oocytes should be a useful system for examining signals responsible for the specific targeting of lysosomal enzymes to lysosomes.

CATHEPSIN D is a lysosomal aspartyl protease that is present in all mammalian cells (1). The biosynthesis and intracellular targeting of cathepsin D have been well studied (6, 12, 15, 16, 30) and have helped to define the posttranslational modifications that are characteristic of many lysosomal enzymes. Biosynthetic studies have demonstrated that cathepsin D is synthesized in the rough endoplasmic reticulum as a preproprotein (6, 30), with the signal peptide removed in this organelle. Procathepsin D, with reported M_r of 46,000–53,000 D (17), is then translocated to the Golgi apparatus where its high mannose-type oligosaccharides may acquire phosphomannosyl residues (15). These mannose 6-phosphate (Man-6-P)[1] residues are recognized by specific receptors (Man-6-P receptor) in the Golgi complex, resulting in sorting of lysosomal enzymes from secretory proteins and subsequent targeting of lysosomal enzymes to lysosomes (3). Some of the procathepsin D molecules escape this segregation and are secreted. The majority of the cathepsin D, however, is targeted to lysosomes and undergoes further proteolytic cleavages. The first cleavage is initiated in prelysosomal compartments (12) and involves removal of the 44 amino acid propiece (6) to generate an intermediate of reported M_r 44,000–47,000 D (17). A second cleavage, occurring in lysosomal compartments, processes the intermediate to a two-chain form with noncovalently associated subunits of M_r 31,000 and 14,000 D (17). Very late in the biosynthesis, the M_r 31,000-D chain undergoes a further M_r ~1,000-D decrease in mass, which most likely represents proteolytic trimming at both its carboxyl and amino termini (5, 8).

The acquisition of Man-6-P residues by lysosomal enzymes is the determining step for the pathway that results in the segregation of these hydrolases into lysosomes. This Man-6-P recognition marker is generated by the sequential action of two enzymes (13). First, *N*-acetylglucosamine-1-phosphate is transferred to selected mannose residues on lysosomal enzymes, giving rise to a phosphodiester intermediate. The *N*-acetylglucosamine is then removed by a second enzyme to expose the Man-6-P monoester signal. The first reaction is catalyzed by UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosaminylphosphotransferase (phosphotransferase) (19, 28). This enzyme is able to selectively phosphorylate lysosomal enzymes over nonlysosomal glycoproteins containing similar oligosaccharides by recognition of a protein domain that is common to many lysosomal enzymes (25, 27). However, the identity of this common protein domain is unknown.

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1. *Abbreviations used in this paper*: endo H, endo-β-*N*-acetylglucosaminidase H; Glc-6-P, glucose-6-phosphate; Man-6-P, mannose-6-phosphate; MBS, modified Barth's saline; MVB, multivesicular body; NAG, β-hexosaminidase; phosphotransferase, UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosaminylphosphotransferase; VG, vitellogenin.

As a first step towards understanding the lysosomal protein recognition domain, we have previously isolated a cDNA clone containing the complete protein-encoding region for human cathepsin D (8). We next sought to express the cDNA in an appropriate system which could be conveniently used to test mutations generated in specific regions of the coding sequence. We report here the expression of the human cathepsin D cDNA in *Xenopus laevis* oocytes and demonstrate that the cathepsin D acquires phosphomannosyl residues and is targeted to lysosomes.

Materials and Methods

Antiserum

Human placental cathepsin D was purified to homogeneity by previously described methods (33) and injected with adjuvants into rabbits. After two booster injections, serum was collected and titered by immunoprecipitation of iodinated cathepsin D.

Plasmids/Oocyte Expression

A cDNA clone for human cathepsin D has been previously described (8). G/C homopolymer tails were removed from 5′ and 3′ ends of the clone by Bal 31 digestion (New England Biolabs, Beverly, MA) followed by DNA sequencing (32) to determine the extent of digestion. The cDNA was then cloned into the expression vector pSP64 (Promega Biotec, Madison, WI).

RNA transcripts were synthesized in vitro from cloned cDNAs with SP6 polymerase (New England Biolabs) as described by Melton et al. (26) except that GTP was reduced to 0.1 mM and RNAs were capped by transcription in the presence of 0.5 mM G(5′)ppp(3′)G (Pharmacia Fine Chemicals, Piscataway, NJ). The amount of transcript produced was monitored by incorporation of [α-^{3}H]UTP. After transcription, RNAs were precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water.

Stage V-VI oocytes were obtained from large *Xenopus laevis* females (Nasco, Fort Atkinson, WI) and maintained in modified Barth's saline (MBS) at 19°C as described (2). Oocytes were injected with 50 nl of RNA (150–200 ng/μl) and then stored overnight in MBS to allow removal of damaged oocytes prior to labeling. For labeling with [^{35}S]methionine (>1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL), oocytes were incubated in groups of 10–20 with 10 μl/oocyte of MBS containing 5% fetal bovine serum and 1 mCi/ml [^{35}S]methionine. For labeling with [2-^{3}H]mannose (14 Ci/mmol, ICN, St. Louis, MO), 200 injected oocytes were incubated for 48 h in 0.5 ml of MBS containing 5 mCi/ml [2-^{3}H]mannose. At the termination of incubations, oocytes and media were separately collected, rapidly frozen on dry ice, and stored at −20°C.

Immune Precipitations

Frozen, labeled oocytes were homogenized in 40 μl/oocyte of 50 mM imidazole, pH 7, 150 mM NaCl, 5 mM Na β-glycerophosphate, 1% Triton X-100, 0.2 TIU/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1:1000 antiprotease mix (buffer I), and centrifuged in a eppendorf microfuge for 2 min at 4°C to remove insolubles. The antiprotease mix consisted of antipain, chymostatin, leupeptin, and pepstatin (each at 2 mg/ml) and aprotinin (10 TIU/ml) in 50% DMSO.

Equivalent samples (corresponding to two oocytes) of cell extract and medium samples were diluted into 0.5 ml of immunoprecipitation buffer (0.1 M KCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% SDS, 1% sodium deoxycholate, 1 mM PMSF, 0.1 M Tris-HCl, pH 8) and incubated with anticathepsin D serum or nonimmune serum for 4–18 h at 4°C. Protein A-Sepharose (Sigma Chemical Co.) was then added and allowed to incubate for 1–2 h at 4°C. The resin was sedimented and washed four times with 1 ml of 1% Triton X-100, 0.1 M Tris-HCl, pH 8, 100 mM NaCl, 0.2 TIU/ml aprotinin, 1 mM PMSF followed by two washes with 0.1 M Tris-HCl, pH 8. Immunoprecipitated proteins were dissociated from the resin by heating for 5 min at 100°C in SDS gel sample buffer, electrophoresed through a SDS/polyacrylamide gel (SDS-PAGE) (23) under nonreducing conditions, and fluorographed using ENhance (New England Nuclear, Boston, MA). When indicated, the appropriate regions of the dried gel were excised and solubilized with 90% NCS tissue solubilizer (Amersham Corp.), and radioactivity was measured by liquid scintillation counting according to the manufacturer's instructions.

Man-6-P Receptor Affinity Chromatography

Bovine liver 215-kD Man-6-P receptor was purified and linked to Affigel 15 as previously described (36). Columns were equilibrated in buffer containing 50 mM imidazole, pH 6.5, 150 mM NaCl, 5 mM Na β-glycerophosphate, 0.05% Triton X-100, and 1:1000 antiprotease mix (buffer II). All manipulations were performed at 4°C.

Cell extract and medium samples were diluted into 0.4 ml of buffer II and applied to a 1.75-ml Man-6-P receptor column. The column was washed with buffer II until no further radioactivity was detected in the buffer run through. The column was then eluted sequentially with 2 mM glucose 6-phosphate (Glc-6-P) in buffer II followed by 5 mM Man-6-P in buffer II. Fractions corresponding to the column run-through and Man-6-P-eluted material were separately pooled, adjusted to contain 50 mM Tris-HCl, pH 8, and 1% Triton X-100, and then immunoprecipitated as described above. The Glc-6-P–eluted fractions were free of radioactivity and were not analyzed further.

Oligosaccharide Analysis

[^{35}S]methionine-labeled cathepsin D immunoprecipitates were treated with 1 mU of endo-β-*N*-acetylglucosaminidase H (endo H; Boerhinger Mannheim, Indianapolis, IN) by dilution of SDS-disaggregated immunoprecipitates with 50 mM citrate-phosphate, pH 5.6, to a final SDS concentration of 0.25%. After overnight incubation under a toluene atmosphere at 37°C, proteins were precipitated with trichloroacetic acid, washed with acetone, and dissolved in SDS sample buffer.

Oocytes that were injected with cathepsin D RNA and then labeled with [2-^{3}H]mannose were homogenized in buffer I and applied to a Man-6-P receptor column. Cathepsin D was isolated from the Man-6-P eluate by immunoprecipitation. Glycopeptides were prepared by pronase digestion of the SDS-disaggregated immunoprecipitate and applied to concanavalin A (Con A)-Sepharose columns as previously described (4). The glycopeptides that eluted with 100 mM α-methylmannoside were desalted by chromatography on Sephadex G-25 and then digested with 2 mU of endo H in 0.1 ml of 50 mM citrate-phosphate, pH 5.6. A sample of the endo H digest was diluted with 2 mM Tris, pH 8, and analyzed by QAE-Sephadex chromatography (35). Separate samples were subjected to mild acid hydrolysis (sample diluted into 0.5 ml of 0.01 N HCl and heated 30 min at 100°C) and/or digestion with *Escherichia coli* alkaline phosphatase (a generous gift of Dr. M. Schlesinger, Washington University, St. Louis, MO) as previously described (35) followed by QAE-Sephadex analysis.

Sucrose-Gradient Fractionation

Oocytes were injected with cathepsin D RNA and then incubated with [^{35}S]methionine for 5, 30, or 70 h. Some labeled oocytes were removed after 24 and 64 h and were incubated with 0.5 mg/ml vitellogenin (VG) in MBS for 6 h. For each gradient, 25 oocytes (15–25 labeled oocytes, supplemented with unlabeled oocytes where necessary) were then homogenized in 1 ml of cold homogenization buffer (0.25 M sucrose, 0.5 mM $MgCl_2$, 1 mM EGTA, 5 mM Hepes, pH 7.4) using six to seven slow strokes with the loose-fitting pestle of a Dounce homogenizer (Kontes Co., Evanston, IL). The homogenates were filtered through Nitex nylon screen (52-μm mesh size: Tetko, Inc., Depew, NY) and then applied to the top of linear sucrose gradients as previously described (37). Cathepsin D was immunoprecipitated from gradient fractions by fourfold dilution into immunoprecipitation buffer. β-Hexosaminidase (NAG) was assayed colorimetrically as described by Hall et al. (14) except that all incubation mixtures contained 0.1% Triton X-100.

Lysosomal Enzyme Uptake

Oocytes were isolated by manual dissection or were freed of follicle cells and other ovarian tissues by collagenase dissociation (2). Phosphorylated lysosomal enzymes were isolated from BW5147 PHA^{R}1.8 cell (34) NH_4Cl-induced secretions and iodinated (a generous gift of J. Duncan of this laboratory). Groups of 15–30 oocytes were incubated in 0.3 ml of MBS containing 5% fetal bovine serum and 30,000 cpm ^{125}I-lysosomal enzymes for 6–24 h at 20°C in the presence and absence of 10 mM Man-6-P or 10 mM Glc-6-P. Oocytes were then washed extensively with MBS and counted directly for ^{125}I in a gamma-counter (Beckman Instruments, Inc., Palo Alto, CA).

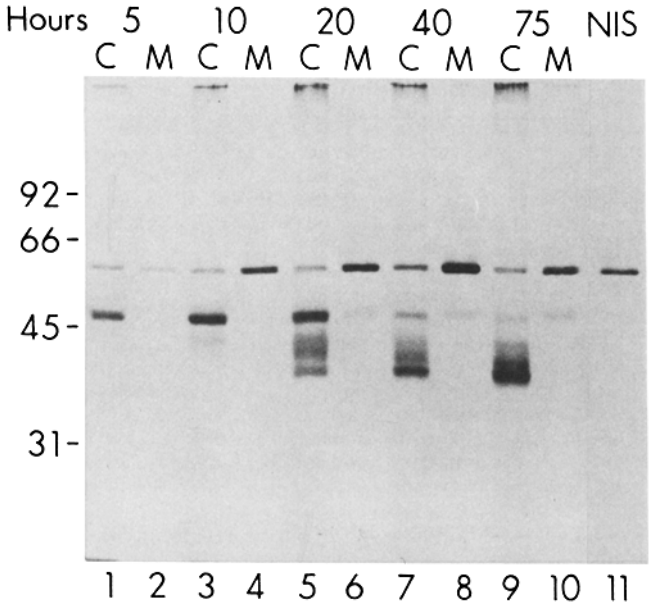

Figure 1. Time course of cathepsin D synthesis in *Xenopus* oocytes. Oocytes were microinjected with cathepsin D RNA and then labeled with [^{35}S]methionine for the indicated times. Equivalent samples of an oocyte detergent extract (*C*) and the medium (*M*) were then immunoprecipitated with anticathepsin D antiserum (lanes *1–10*) or nonimmune serum (lane *11*) and analyzed by SDS-PAGE (10% gel) and fluorography. The band at M_r 59,000 D is nonspecific as it appears in the nonimmune serum control (lane *11*; same band present in cell and medium samples). Molecular size standards are in kilodaltons.

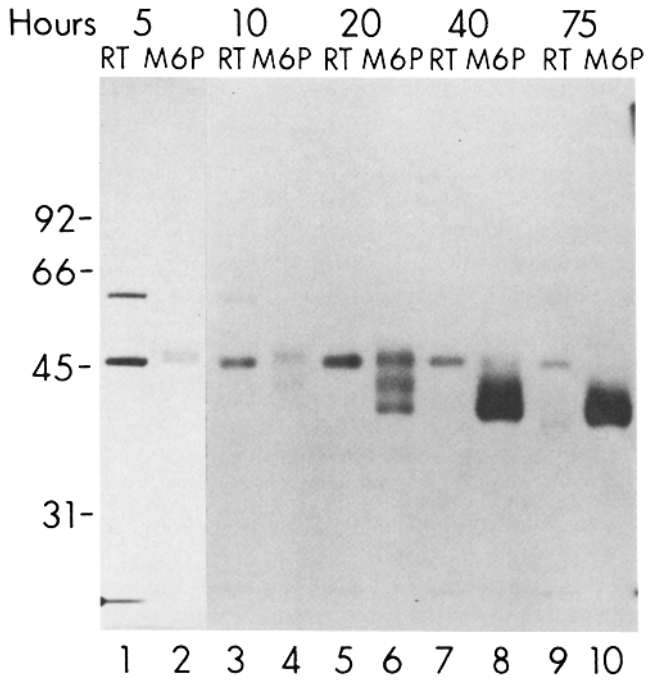

Figure 2. Binding of [^{35}S]methionine-labeled cathepsin D to the Man-6-P receptor. Oocyte detergent homogenates (Fig. 1) from the indicated times were applied to a Man-6-P receptor column and specifically eluted with 5 mM mannose 6-P. Fractions corresponding to the column run-through (*RT*) and mannose 6-P eluate (*M6P*) were immunoprecipitated with anticathepsin D antiserum and analyzed by SDS-PAGE and fluorography.

Results

Expression of Cathepsin D in Xenopus Oocytes

Oocytes were microinjected with a cathepsin D RNA message generated by in vitro transcription of the cloned cDNA inserted behind the bacteriophage SP6 promoter. Injected oocytes were incubated with [^{35}S]methionine over a period of 75 h and cathepsin D associated with the oocytes and in the medium was immunoprecipitated with anticathepsin D antiserum (note that incubation past 20 h constitutes a chase in this system [2]). After 5 h of incubation, an M_r 47,000-D protein was immunoprecipitated from the oocyte homogenate and first detected in the medium at 20 h (Fig. 1, lanes *1* and *6*, respectively). By 75 h of incubation, ~5% of the cathepsin D molecules synthesized by the oocyte were secreted (Fig. 1, lane *10*). The majority of the cathepsin D, however, remained cell-associated and was slowly processed to an M_r 39,000-D protein with apparent processing intermediates at M_r 43,000–41,000 D (Fig. 1).[2] The disappearance of the M_r 47,000-D protein and sequential appearance of the M_r 43,000–41,000-D processing intermediates leading to the M_r 39,000-D mature protein indicates a precursor–product relationship for these forms. The processed forms were not detected in the medium, consistent with the intracellular location of cathepsin D processing as defined in mammalian cells (17). These immunoprecipitable proteins were not present in anticathepsin D antiserum precipitates of noninjected oocytes (data not shown) or in nonimmune antiserum precipitates of cathepsin D-injected oocytes (Fig. 1, lane *11*).

Proteins of M_r 31,000 and 14,000 D, corresponding to the heavy and light chains of cathepsin D, respectively, were only detected when the gels were overexposed (data not shown; M_r 14,000 D detected on higher-percentage gels). The amount of these forms detected was low (not >1% of all cathepsin D molecules) and highly variable and may actually represent minor proteolysis at a sensitive site during homogenization rather than a true processing product of the cell.

Phosphorylation of Cathepsin D

In mammalian cells, newly synthesized cathepsin D acquires phosphomannosyl residues (15), which are required for its targeting to lysosomes by the Man-6-P receptor. To determine whether cathepsin D synthesized by oocytes also acquires Man-6-P residues, [^{35}S]methionine-labeled oocyte homogenate and media samples were applied to Man-6-P receptor columns. The column was washed with buffer until no more radioactivity eluted, then with 2 mM Glc-6-P, a sugar phosphate that does not interact with the receptor, and finally with 5 mM Man-6-P, a competitive inhibitor for receptor binding. The material in the column run-through and the Man-6-P eluate was then immunoprecipitated with

2. The electrophoretic mobilities differed when samples were prepared under reducing conditions. The M_r 47,000-D form did not change mobility. The processing intermediates were not resolved from each other and ran as one band with M_r 46,000 D. The M_r 39,000-D form electrophoresed with apparent M_r 45,000 D.

Table I. Quantification of Cathepsin D Phosphorylation

Time	Sample	Run-through	Man-6-P eluate	Percent phosphorylated
h		*cpm cathepsin D*		%
5	Cells	2,248	416	16
10	Cells	2,287	1,156	34
20	Cells	3,490	12,510	78
40	Cells	2,068	25,480	93
75	Cells	547	23,765	98
75*	Medium	453	790	64

Appropriate regions of the dried gel shown in Fig. 2 were excised and the radioactivity present was measured by liquid scintillation counting.
* Determined from a separate experiment, not shown in Fig. 2.

anticathepsin D antiserum. As shown in Fig. 2, cathepsin D bound to the receptor column and was specifically eluted with Man-6-P, indicating the presence of the Man-6-P marker. The extent of receptor binding of the intracellular cathepsin D increased from 16 to 98% between 5 and 75 h of incubation with [^{35}S]methionine (Fig. 2 and Table I). 64% of the secreted cathepsin D also bound to the receptor column (Table I). Although the level of cathepsin D secretion varied between 5 and 25% of the total cathepsin D molecules synthesized by oocytes from different animals (data not shown), these results demonstrate that the phosphorylation of cathepsin D by oocytes is very efficient, with a total level of phosphorylation (cells and medium material) of 93–97%. In addition, the fractions which bound to the Man-6-P receptor column initially contained the precursor form (Fig. 2, lane *2*) and then, with increasing time of incubation, the processed forms of cathepsin D were detected; in contrast, the column run-through contained only the precursor form (Fig. 2). This indicates that processing occurs after phosphorylation. This is consistent with the sequence of events characterized for cathepsin D in mammalian cells where phosphorylation occurs in the early Golgi apparatus followed by proteolytic cleavages subsequent to targeting to prelysosomal and lysosomal organelles (12, 13, 17).

Endogenous proteins in *Xenopus* oocytes are also capable of binding to a Man-6-P receptor column. The extent of receptor binding for these proteins increased over the 75-h incubation period at a rate identical to that seen for cathepsin D with a maximum of ~1.0% of total [^{35}S]methionine-labeled endogenous proteins binding to the receptor column (data not shown). Lysosomal enzyme activities for three endogenous lysosomal enzymes were assayed in the receptor column run-through and Man-6-P eluate. The percentage of activity that bound to the column ranged from 24 to 45% (Table II). Thus, lysosomal enzyme activity is associated with the endogenous proteins that bind to the Man-6-P receptor column. In addition, it is apparent that *Xenopus* oocytes do not rapidly degrade the Man-6-P marker, as observed in some mammalian cells (10), in that almost all of the newly synthesized, intracellular cathepsin D still bound to the receptor column at the end point (75 h) of the experiment (Table I). The lower level of receptor binding observed for the endogenous lysosomal enzyme activities (Table II) probably reflects dephosphorylation after extended residence in a lysosomal compartment. The presence of a small amount of processed cathepsin D which does not bind to the receptor column, detected only after 75 h of incubation (Fig. 2, lane *9*), may also reflect this dephosphorylation.

Table II. Lysosomal Enzyme Binding to Phosphomannosyl Receptor Column in Xenopus Oocytes

Enzyme	Run-through	Man-6-P eluate	Percent bound
	nmol product/h		%
β-Hexosaminidase	1,455	455	24
β-Galactosidase	16	13	45
β-Glucuronidase	1.9	0.8	30

Cell extracts were applied to a Man-6-P receptor column. Run-through and Man-6-P eluates were assayed for lysosomal enzymes using 4-methyl-umbelliferone substrates as described (29).

Oligosaccharide Analysis

Cathepsin D immunoprecipitates of the receptor column run-through and Man-6-P eluates from the 20-h time point (Fig. 2, lanes *5* and *6*, respectively) were treated with endo H. Both fractions showed increased mobility on SDS-PAGE after endo H treatment (Fig. 3), indicating that the oligosaccharide chains are of the high-mannose type. The observed M_r 4,000-D decrease for all protein species is consistent with removal of two high-mannose-type oligosaccharide chains, indicating that both asparagine-linked glycosylation signals on cathepsin D (8) are being utilized. In addition, all of the processed forms of cathepsin D shifted independently (Fig. 3, lanes *3* and *4*), indicating that the different electrophoretic mobilities are not due to oligosaccharide processing and most likely represent proteolytic cleavage.

Further analysis of the oligosaccharides of cathepsin D was undertaken to directly demonstrate the presence of phosphomannosyl residues. As *Xenopus* oocytes inefficiently incorporate mannose into oligosaccharides (P. Faust, unpublished observations), a large number of oocytes were microinjected with cathepsin D RNA and labeled continuously with [2-

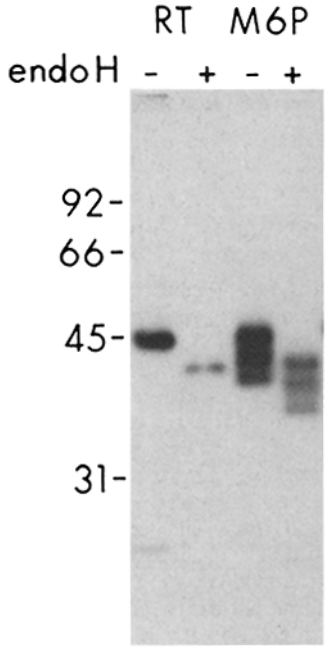

Figure 3. Sensitivity of cathepsin D to endo-β-*N*-acetylglucosaminidase H. Cathepsin D was isolated by immunoprecipitation from the Man-6-P receptor column run-through (*RT*) and Man-6-P eluates (*M6P*) at the 20-h time point (Fig. 2, lanes *5* and *6*). Immunoprecipitates were then incubated with or without endo-β-*N*-acetylglucosaminidase H (*endo H*) followed by SDS-PAGE and fluorography.

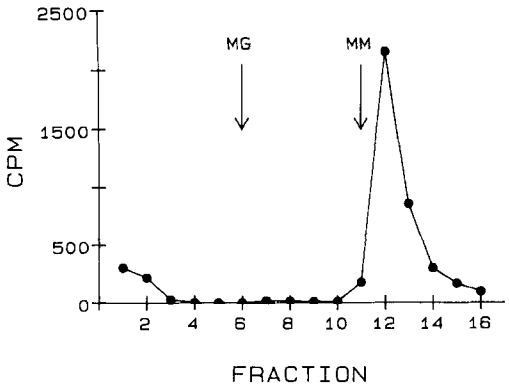

Figure 4. Con A-Sepharose chromatography of cathepsin D glycopeptides. Oocytes were injected with cathepsin D RNA and then labeled with [2-^{3}H]mannose for 48 h. The oocytes were homogenized and applied to a Man-6-P receptor column. Cathepsin D was then isolated from the Man-6-P eluate by immunoprecipitation. Glycopeptides generated by pronase digestion of the SDS-disaggregated immunoprecipitate were applied to a Con A-Sepharose column. The column was eluted with 10 mM α-methylglucoside (*MG*) followed by 100 mM α-methylmannoside (*MM*).

^{3}H]mannose for 48 h. The cellular homogenate was then applied to the Man-6-P receptor column and cathepsin D isolated by immunoprecipitation from the Man-6-P eluate. Similar immunoprecipitates so obtained from [^{35}S]methionine-labeled samples were found to be at least 90% pure cathepsin D with respect to radioactivity by SDS-PAGE (see, for example, Fig. 2). After immunoprecipitation, the cathepsin D was digested with pronase to generate glycopeptides, which were then applied to a column of Con A-Sepharose and eluted sequentially with 10 mM α-methylglucoside and 100 mM α-methylmannoside. Under these conditions, complex-type asparagine-linked oligosaccharides either pass through the column or are eluted with α-methylglucoside, whereas high-mannose-type oligosaccharides require α-methylmannoside for elution (4). 90% of the radioactivity bound to the column and required α-methylmannoside for elution (Fig. 4), consistent with the endo H analysis (Fig. 3) which demonstrated high-mannose-type oligosaccharides on cathepsin D.

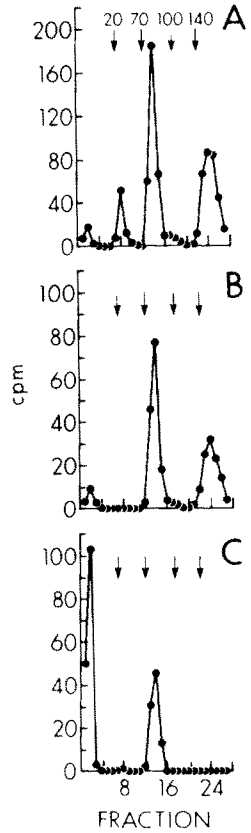

Figure 5. QAE-Sephadex analysis of cathepsin D oligosaccharides. The α-methylmannoside–eluted glycopeptides (Fig. 4) were desalted on Sephadex-G25 and digested with endo H to release the oligosaccharides. Samples of the endo H reaction were applied to analytical QAE-Sephadex columns after (*A*) no treatment, (*B*) mild acid treatment, or (*C*) mild acid + alkaline phosphatase treatment. *20*, *70*, *100*, and *140* refer to millimolar concentration of NaCl in elution buffer.

The high-mannose glycopeptides were treated with endo H to release the oligosaccharides which were then analyzed for the presence of phosphomannosyl residues by QAE-Sephadex chromatography (35). Under the conditions used, neutral oligosaccharides pass through the resin while oligosaccharides with one, two, three, or four net negative charges bind and are eluted with 20, 70, 100, and 140 mM NaCl, respectively. As shown in Fig. 5 *A*, 96% of the radioactivity interacted with the resin and eluted at positions characteristic for oligosaccharides with one phosphodiester (20 mM NaCl), one phosphomonoester (70 mM NaCl), and two phosphomonoesters (140 mM NaCl). Mild acid hydrolysis, which will remove the *N*-acetylglucosamine from phosphodiester residues, caused the 20 mM peak of radioactivity to disappear and shift to the 70 mM position (Fig. 5 *B*; the shift to 70 mM is apparent when percent counts per minute in each peak are calculated), identifying this material as an oligosaccharide with a single phosphodiester. The combined treatments of mild acid followed by alkaline phosphatase digestion caused 63% of the radioactivity to run through the column, characteristic of neutral oligosaccharides (Fig. 5 *C*). This confirms that the material eluting at 140 mM, and at least part of that at 70 mM, contains oligosaccharides with phosphomonoesters. Insufficient material was available to confirm the nature of the radioactivity eluting at 70 mM NaCl after these combined treatments. It most likely represents incomplete digestion by the alkaline phosphatase as other samples, subsequently shown to contain phosphomonoesters, were also incompletely digested with the enzyme preparation used for these experiments. A similar analysis of the endogenous glycoproteins of *Xenopus* oocytes also demonstrated phosphorylated oligosaccharides by QAE-Sephadex chromatography; Man-6-P residues were directly demonstrated by total acid hydrolysis of endo H-released oligosaccharides followed by paper chromatography (9; data not shown).

Sucrose-Gradient Fractionation

A previous study (37) on characterization of lysosomes from stage V-VI *Xenopus* oocytes by sucrose-gradient fractionation demonstrated two distinct populations of lysosomes: (*a*) a subpopulation of yolk platelets, termed light yolk platelets, which contain the majority of the lysosomal enzyme activity, and (*b*) a population of "light lysosomes" which appear as a broad band sedimenting between 1.12 and 1.18 g/ml sucrose. The light yolk platelets are an apparent precursor to heavy yolk platelets, which are the final destination of the majority of yolk proteins (37). However, little hydrolase activity is associated with the heavy yolk platelets. Stage V-VI oocytes, injected with cathepsin D RNA, were labeled with

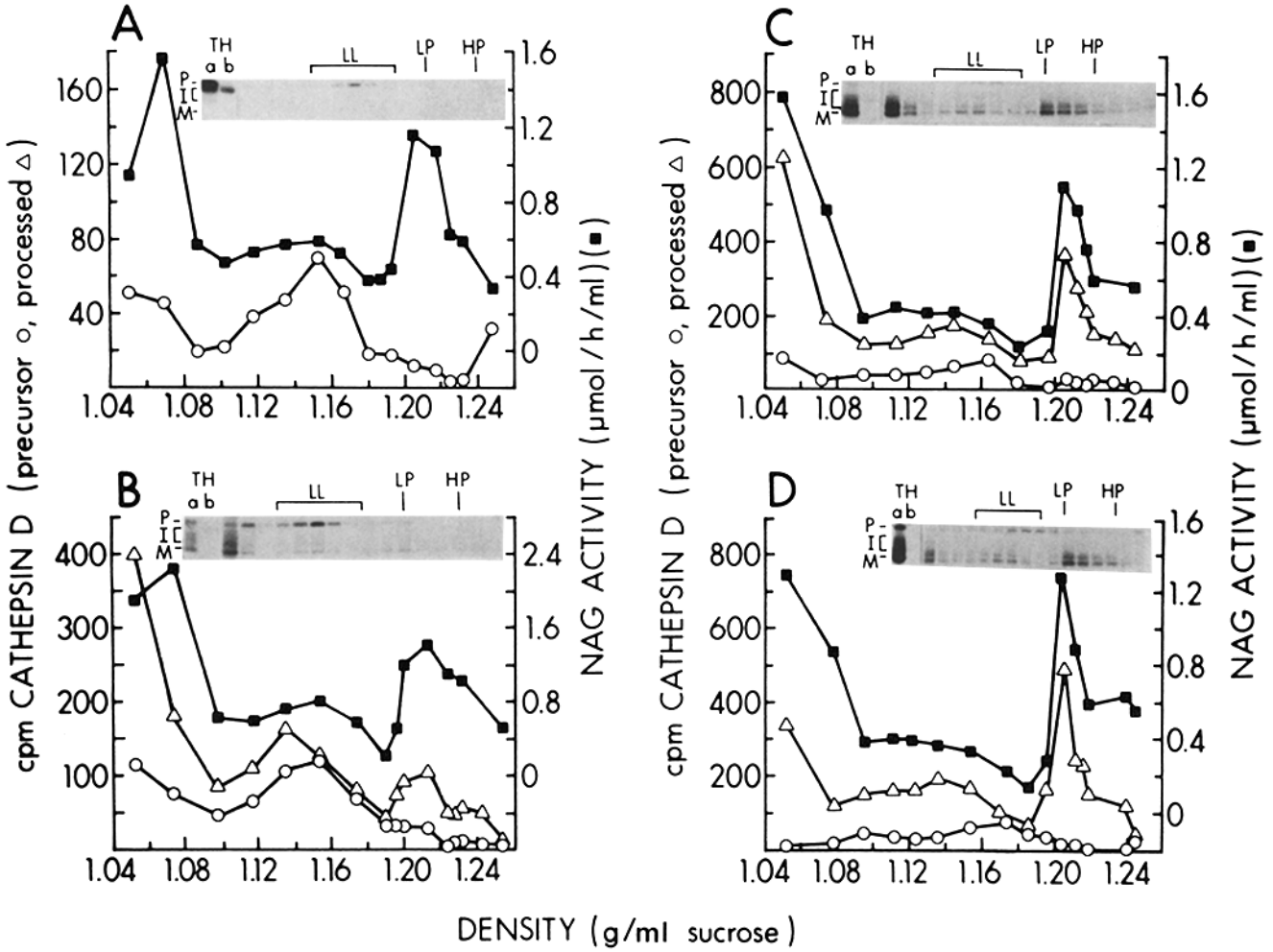

Figure 6. Sucrose-gradient fractionation of oocytes injected with cathepsin D RNA. Injected oocytes were labeled with [^{35}S]methionine and incubated for a total of (*A*) 5, (*B*) 30, or (*C, D*) 70 h. Oocytes in *B* and *C* were removed from the labeling medium after 24 and 64 h, respectively, and incubated with 0.5 mg/ml VG for 6 h. Oocytes were then homogenized and applied to linear sucrose gradients for analysis of the NAG and cathepsin D distribution. *Insets*: The cathepsin D distribution was determined by immunoprecipitation of odd-numbered gradient fractions except in the light yolk platelet (*LP*) and heavy yolk platelet (*HP*) region where every fraction was analyzed. *TH*, total homogenate applied to sucrose gradient immunoprecipitated with (*a*) anticathepsin D serum or (*b*) nonimmune serum. The positions of precursor (*P*), intermediate (*I*), and mature (*M*) forms of cathepsin D are indicated. Characteristic regions of the gradient are indicated for the gel lanes: *LL*, light lysosome region, 1.12–1.18 g/ml; *LP*, light yolk platelets, 1.21 g/ml; *HP*, heavy yolk platelets, 1.23 g/ml sucrose (note that the gel lanes do not necessarily line up with the densitities indicated in the graph). *Graphs*: NAG activity (■) was assayed using *p*-nitrophenyl-*N*-acetylglucosamine as a substrate. The cathepsin D distribution was quantified by excising appropriate regions of the dried gel (*inset*) and measuring the radioactivity by liquid scintillation counting. Precursor (○) and processed (△; intermediates + mature) forms were excised separately.

[^{35}S]methionine and then fractionated on identical sucrose gradients in order to follow the compartmental distribution of cathepsin D during its biosynthesis and to determine whether it is targeted to the light yolk platelets. The distribution of the endogenous NAG activity was determined for comparison. In agreement with previous findings, a large peak of NAG activity is associated with the light yolk platelet compartment, which bands at 1.21 g/ml sucrose, and a second peak of NAG activity is found in the broad band corresponding to the light lysosome region (Fig. 6). The heavy yolk platelets, which band at 1.23 g/ml, have little associated NAG activity. The large amount of nonsedimentable NAG activity observed in these gradients most likely represents release of NAG during homogenization, predominantly from the fragile light yolk platelets (37).

The distribution of cathepsin D across the gradients was determined by immunoprecipitation followed by SDS-PAGE and was quantified by solubilization and scintillation counting of appropriate regions of the dried gels (Fig. 6). After 5 h of incubation, a time at which some phosphorylation but no proteolytic processing of cathepsin D has occurred (Fig. 2), the precursor M_r 47,000-D form of cathepsin D sedimented as a broad band between 1.12 and 1.18 g/ml with a distinct peak at 1.154 g/ml (Fig. 6 *A*). Precursor was not detected in the light yolk platelet region at this time. After 30 h of incubation (Fig. 6 *B*), most of the precursor remains at

Table III. Lysosomal Enzyme Endocytosis by Xenopus Oocytes

Oocyte isolation	Oocytes per well	Time	Addition to assay	^{125}I	Man-6-P-specific uptake
	n	*h*		*cpm*	
Collagenase treated	15	6	–	676	613
			Man-6-P	63	
	15	24	–	3,236	3,006
			Man-6-P	230	
Manually dissected	20	6	–	1,139	910
			Man-6-P	229	
	20	24	–	3,828	3,193
			Man-6-P	635	
	30	24	–	7,524	6,079
			Man-6-P	1,445	
			Glc-6-P	15,370	

Oocytes were isolated by manual dissection or collagenase dissociation and then incubated with ^{125}I-lysosomal enzymes in the presence or absence of Man-6-P or Glc-6-P. After the indicated time, the oocytes were washed and counted directly for ^{125}I in a gamma-counter. All determinations were performed on duplicate wells of oocytes.

1.154 g/ml. In contrast, processed forms of cathepsin D (intermediates and mature form) appear in two distinct regions: one peak at 1.135 g/ml, in the light lysosome region of the gradient and somewhat separate from the precursor peak, and a second peak at 1.21 g/ml, in the light yolk platelet band. Small amounts of precursor were also detected in the 1.21 g/ml region of the gradient. By 70 h, even more of the cathepsin D sedimented with the light yolk platelets (Fig. 6, *C* and *D*) and it is in a predominantly processed form. The distribution of cathepsin D at this time is identical to that of NAG. As observed for NAG, there is little cathepsin D associated with the heavy yolk platelets at 1.23 g/ml. In addition, to determine whether endocytosis of the yolk protein VG was required for cathepsin D to reach the light yolk platelet compartment, some oocytes were incubated with VG for 6 h before homogenization. This incubation had no discernable effect on the distribution of cathepsin D in these gradients at the 30- (30 h without VG not shown) and 70-h time points (Fig. 6, *C* and *D*, with and without VG, respectively). The large amount of cathepsin D which was nonsedimentable in these gradients was probably released largely from damaged yolk platelets since there was a greater release of processed forms compared to the precursor. These gradients have defined three distinct compartments traversed by cathepsin D during its biosynthesis: a 1.154-g/ml band which is enriched for precursor, a 1.135-g/ml band which is enriched for processed forms, and the 1.21-g/ml light yolk platelet band.

Lysosomal Enzyme Uptake

In some simple eukaryotic organisms, such as the soil ameba *Acanthamoeba castellani* and the slime mold *Dictyostelium discoideum*, the phosphotransferase enzyme is present (24) but it has not yet been determined whether these cells utilize the Man-6-P marker for intracellular targeting of lysosomal hydrolases. It was of interest, then, to determine whether oocytes have a Man-6-P receptor which could function to target phosphorylated lysosomal enzymes to lysosomes. To investigate this, phosphorylated lysosomal enzymes were isolated from secretions of a murine lymphoma cell line and iodinated. Oocytes were then incubated with the ^{125}I-lysosomal enzymes in the presence and absence of Man-6-P. As shown in Table III, oocytes isolated by manual dissection or collagenase dissociation (a procedure that releases the adherent follicle cells) were capable of taking up ^{125}I-lysosomal enzymes from the medium and this uptake was inhibited by Man-6-P. Glc-6-P did not inhibit uptake (the increased uptake in the presence of Glc-6-P is not understood but is consistently observed). The linear increase in cell-associated radioactivity with time suggests that the lysosomal enzymes are being internalized and accumulating within the oocyte. Manually dissected oocytes endocytosed similar levels of radioactivity as collagenase-treated oocytes, indicating that the uptake was due to the oocytes and not to follicle cell contamination. Although oocytes can take up serum proteins by fluid-phase endocytosis (40), the demonstrated inhibition by Man-6-P indicates that the lysosomal enzyme uptake is a specific receptor-mediated process. In addition, preliminary studies of [^{35}S]methionine-labeled oocytes have demonstrated oocyte proteins of M_r ~215,000 and 43,000 D which are capable of specifically binding to phosphomannan-Sepharose in a cation-independent and cation-dependent manner, respectively (N. Dahms and S. Kornfeld, unpublished observations). Thus oocytes have two Man-6-P–binding proteins which have properties similar to the two Man-6-P receptors characterized in mammalian cells (20, 31).

This specific uptake of lysosomal enzymes by oocytes raised the possibility that the observed targeting of cathepsin D to lysosomes (Fig. 6) may occur by a secretion-recapture mechanism rather than a direct intracellular pathway. To investigate this possibility, oocytes were injected with cathepsin D message and then labeled with [^{35}S]methionine in the presence or absence of Man-6-P or Glc-6-P. The addition of Man-6-P or Glc-6-P to the labeling medium had no effect on the distribution between cells and medium and the intracellular processing of cathepsin D (data not shown). This finding is consistent with studies in mammalian cells which indicate that secretion-recapture plays a minor role in the targeting of newly synthesized lysosomal enzymes (3, 17).

Discussion

The data presented in this paper demonstrate that human cathepsin D expressed in *Xenopus laevis* oocytes acquires phosphomannosyl residues and is targeted to lysosomes. In agreement with biosynthetic studies on cathepsin D in mammalian cells (6, 12, 15, 16, 30), most of the cathepsin D in oocytes remains intracellular and undergoes proteolytic cleavage upon delivery to lysosomal organelles. The cathepsin D that is secreted (5–25%) is the higher molecular mass precursor form, presumably procathepsin D (6). 93–97% of the cathepsin D molecules synthesized by the oocyte were phosphorylated, demonstrating that the oocyte phosphotransferase is capable of efficiently recognizing this mammalian lysosomal enzyme. We have also demonstrated that the nonrelated glycoproteins ovalbumin and α-lactalbumin do not acquire phosphomannosyl residues in the oocyte (7), thus indicating the specificity of the phosphorylation event.

The proteolytic cleavage of cathepsin D in oocytes differed from that previously observed in mammalian cells. First, the mature form in oocytes is a protein of M_r 39,000 D (M_r 45,000 D on reducing gels) rather than the two-chain form with M_r values of 31,000 and 14,000 D (17). In mammalian cells, inhibitors of lysosomal cysteine proteinases block conversion of the single chain enzyme to the mature two-chain form (11). The lysosomal organelles in *Xenopus* oocytes have an unusual enzymatic composition with little proteolytic activity (37). Indeed, cathepsin B, a cysteine protease, was not detected in oocytes (37). The persistence of the single-chain cathepsin D is, therefore, consistent with the unusual content of the oocyte lysosome. Secondly, the proteolytic cleavage of the M_r 47,000-D precursor to the M_r 39,000-D mature form occurred in multiple steps. The size of the total molecular mass decreased and the demonstrated correlation of cleavage with phosphorylation (Fig. 2) and delivery to lysosomal organelles (Fig. 6) is consistent with the cleavage representing removal of the cathepsin D propiece. A study on the activation mechanism of pepsinogen (22), a related aspartyl protease, demonstrated that removal of the propeptide may occur by a direct conversion, with release of the intact propeptide, or by a sequential pathway in which one or more intermediates can be identified. The propeptide of cathepsin D is also postulated to act as an activation segment (6, 18). Hasilik et al. (18) reported that in vitro activation of cathepsin D shifted the M_r from 53,000 to 51,500 D and not to 47,000 D as seen in intact cells. This M_r 51,500-D form may then represent an intermediate in the removal of the propiece, analogous to that seen in oocytes. The reason why these intermediates in cathepsin D biosynthesis are observed in oocytes but not in mammalian cells may relate to the slower rate at which cathepsin D is translocated to lysosomes as well as to differences in the enzymic content of the lysosomal organelles.

The sucrose-gradient analysis demonstrates that cathepsin D is retained in intracellular membranous vesicles and is targeted to lysosomal organelles, the light lysosomes and the light yolk platelets. In the light lysosome region of the gradient, from 1.12 to 1.18 g/ml sucrose, precursor and processed forms band at slightly different densities suggesting that these forms of cathepsin D are in different compartments. The precursor, which designates an early biosynthetic form, bands at 1.154 g/ml sucrose and most likely represents cathepsin D in endoplasmic reticulum and/or Golgi membranes. It has been demonstrated that the proteolytic processing of cathepsin D is initiated in prelysosomal compartments of mammalian cells (12). By analogy, in oocytes the processed cathepsin D, at 1.135 g/ml, may reside in primary lysosomes formed by the Golgi apparatus and/or in multivesicular prelysosomal bodies. The compartment that the cathepsin D next enters is at 1.21 g/ml sucrose, which corresponds to the peak of NAG activity and the position of the light yolk platelets. The cathepsin D in this compartment is predominantly the processed form, as also observed in mammalian lysosomes (12, 17).

Pulse-chase studies with ^{125}I-VG have demonstrated that the ligand is first found in the light yolk platelet region of the gradient before its appearance in the heavy platelets, which are the final destination of yolk proteins (37). In contrast to the light yolk platelets, the heavy platelets have little associated hydrolase activity. In agreement with this finding, little cathepsin D was associated with the heavy platelets at 1.23 g/ml. Inasmuch as immunoprecipitable protein and not enzymatic activity was measured for cathepsin D in this study, this finding suggests that the absence of hydrolytic activity in the heavy platelets is not due to enzyme inactivation. Rather, absence of the enzymes in the heavy yolk platelet compartment may occur either by their degradation in or recycling out of this compartment.

In many cell types, multivesicular bodies (MVBs) appear to be the point at which lysosomal enzymes are introduced into the endocytic pathway (21). MVBs play a central role in the endocytic pathway of the yolk protein precursor VG in *Xenopus* oocytes (38). VG is destined for long-term storage in yolk platelets as protein crystals, and the MVB is the site in which the condensation and crystallization of the yolk proteins is initiated (38). The MVB also appears to be the site in which VG processing to mature yolk proteins begins, since MVBs as light as 1.10–1.12 g/ml sucrose appear to contain cleaved VG, suggesting that protease activity is present in these organelles (39). In the studies reported here, processed forms of cathepsin D were found at densities from 1.12 to 1.18 g/ml, and their presence there was unaltered by VG endocytosis. Because incoming VG rapidly increases the density of the endocytic organelles containing this ligand, this observation indicates that the lighter density compartments containing processed cathepsin D are inaccessible to newly internalized VG, and thus are distinct from endosomal organelles of the VG endocytic pathway (39). A class of MVBs that do not fuse with endosomes containing newly internalized VG has been described (39), although direct morphological localization will be required to confirm that cathepsin D or other hydrolases are found in these organelles.

Introduction of lysosomal enzymes into the VG endocytic pathway at the level of MVBs or other endosomes would initially place the hydrolases in a relatively low-density organelle. On the sucrose gradients used here, ligand-containing organelles from 1.10 to 1.20 g/ml consist predominantly of MVBs containing yolk proteins in varying stages of condensation and crystallization (38, 39). Appearance of cathepsin D in light yolk platelets at 1.21 g/ml would require either: (*a*) transformation of MVBs into light yolk platelets by the accumulation and crystallization of yolk proteins within them, or *b*) fusion of MVBs or primary lysosomes with preexisting light yolk platelets. In this study, concurrent VG endocytosis was not required for appearance of cathepsin D in platelets, indicating that the first alternative is incorrect. Thus it appears that either fusion of primary lysosomes with MVBs and their eventual delivery to platelets occurs even when MVBs contain no VG, or that a direct pathway for lysosomal enzyme delivery to light platelets exists which does not involve endosomal MVBs (i.e., direct fusion of primary lysosomes with light yolk platelets).

Xenopus oocytes have been utilized for the expression of a variety of foreign proteins, and they are capable of performing many posttranslational modifications as well as directing the correct cellular location of the expressed proteins (2). The data presented here establish that *Xenopus* oocytes have the phosphotransferase enzyme required for generation of the Man-6-P marker. The ability of the oocytes to endocytose phosphorylated lysosomal enzymes also indicates the presence of a Man-6-P receptor that could serve to target the phosphorylated lysosomal enzymes to lysosomes.

Thus, the entire Man-6-P dependent targeting pathway is present in the oocyte. Given the efficiency of cDNA expression utilizing transcription-linked translation, *Xenopus* oocytes should prove to be a versatile system for examining the protein determinants on lysosomal enzymes that are necessary for targeting to lysosomes.

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