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Abnormal Glycosylation of Procathepsin L Due to N-terminal Point Mutations Correlates with Failure to Sort to Lysosomes*

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Richard L. Chapman, Susan E. Kane‡, and Ann H. Erickson§

From the Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599 and the ‡Department of Cell and Tumor Biology, Division of Surgery, City of Hope National Medical Center, Duarte, California 91010

A single point mutation in the lysosomal proenzyme receptor-inhibiting sequence near the N terminus of mouse procathepsin L can result in glycosylation of a normally cryptic site near its C terminus. When alanine replaced His^{36}, Arg^{38}, or Tyr^{40}, the nascent chain of the mutant protein cotranslationally acquired a high mannose oligosaccharide chain at Asn^{268}. In contrast, when alanine replaced Ser^{34}, Arg^{37}, or Leu^{39}, this second carbohydrate chain was not added. This alternating pattern of abnormal glycosylation suggested that propeptide residues 36–40 normally assume an extended conformation having the side chains of residues 36, 38, and 40 facing in the same direction. When tyrosine conservatively replaced His^{36} or lysine replaced Arg^{38}, Asn^{268} was not glycosylated. But the procathepsin L mutant having phenylalanine in place of Tyr^{40} was glycosylated at Asn^{268}, which indicates that the hydrogen bond between the hydroxyl group of Tyr^{40} and the carboxylate group of Asp^{82} is necessary for normal folding of the nascent proenzyme chain.

Mutation of the adjacent α2p (ERININ) helix of the propeptide or addition of a C-terminal epitope tag sequence to procathepsin L also induced misfolding of the proenzyme, as indicated by addition of the second oligosaccharide chain. In contrast, the propeptide mutation KAKK99–102AAAA had no effect on carbohydrate modification even though it reduced the positive charge of the proenzyme.

Misfolded mutant mouse procathepsin L was not efficiently targeted to lysosomes on expression in human HeLa cells, even though it acquired phosphate on mannose residues. The majority of the mutant protein was secreted after undergoing modification with complex sugars. Similarly, epitope-tagged mouse procathepsin L was not targeted to lysosomes in homologous mouse cells but was efficiently secreted. Since production of mature endogenous protease was not reduced in cells expressing the tagged protein, the tagged protein did not compete with endogenous procathepsin L for targeting to lysosomes.

The lysosomal proenzyme receptor (LPR)[1] is a 43-kDa integral membrane protein that binds mouse procatL at pH 5 to microsomal membranes from mouse fibroblasts (1). A short synthetic peptide containing a 9-residue sequence from mouse procathepsin L (procatL) inhibits the binding of the procatL to the LPR, but a scrambled version of this peptide does not (2). To study the physiological effects of inhibiting pH-dependent membrane association, we made over a dozen mutants of mouse procatL having from one to four substitutions in the LPR-inhibiting sequence (LIS, Lys-Ser-Thr-His-Arg-Arg-Leu-Tyr-Gly, residues 33–41, preprocatL numbering (2)), which begins 16 residues from the N terminus of procatL.

During expression of these mouse mutants in human HeLa cells, we found that certain LIS residues were critical for normal glycosylation of the proenzyme. Mouse procatL contains two potential sites (Asn^{221} and Asn^{268}) for cotranslational attachment of an asparagine-linked high mannose oligosaccharide chain (3), but it is normally glycosylated only at Asn^{221} (4). We have found that mutation of any one of several LIS residues, which are located in the N-terminal 10% of the 317-residue procatL chain, sufficiently changes the folding of the nascent chain during translation that it is also glycosylated at Asn^{268}, a normally cryptic site located in the C-terminal 20% of the procatL chain. Secretion of a protein is often used to indicate that the protein is correctly folded because grossly misfolded proteins are normally degraded in the endoplasmic reticulum (ER) (5). Yet protein misfolding may be missed if secretion is used as the only indicator of correct protein conformation. In this study, the addition of an oligosaccharide chain to a normally cryptic site serves as an indicator that a subtle perturbation of the normal pathway of protein folding has occurred.

EXPERIMENTAL PROCEDURES

Materials—Reagents used include the following: [^{35}S]methionine (Trans^{35}S-label; 850–950 Ci/mmol) and [^{32}P]orthophosphate (400–800 mCi/ml) from ICN Biomedicals, Costa Mesa, CA; Protein-A Sepharose CL4B from Pharmacia Biotech Inc.; Dulbecco's modified Eagle's medium, Opti-MEM, LipofectAMINE, and fetal bovine serum from Life Technologies, Inc.; aprotinin, dithiothreitol, endo-β-*N*-acetylglucosaminidase H (EndoH), *N*-glycosidase F, HEPES, pepstatin, and phenylmethanesulfonyl fluoride from Boehringer Mannheim; enhanced chemiluminescence Western blotting detection reagents and donkey anti-rabbit and goat anti-mouse IgG conjugated to horseradish peroxidase from Amersham Corp.; Immobilon-P from Millipore, Bedford, MA; brefeldin A from Epicentre Technologies, Madison, WI; Muta-Gene *in vitro* mutagenesis kit and prestained molecular mass marker proteins from Bio-Rad; eukaryotic expression vector pSG5 from Stratagene, La Jolla, CA; prokaryotic expression vector pET-27b and HSV-Tag monoclonal antibody from Novagen, Madison, WI; plasmid purification columns from Qiagen, Chatsworth, CA; oligonucleotides from Lineberger

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§ To whom correspondence should be addressed. Tel.: 919-966-4694; Fax: 919-966-2852.

[1] The abbreviations used are: LPR, lysosomal proenzyme receptor; EndoH, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; LIS, LPR-inhibiting sequence; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.2; PCR, polymerase chain reaction; procatL, procathepsin L; HSV, herpes simplex virus; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

Nucleic Acids Core Facility, University of North Carolina, Chapel Hill, NC; restriction endonucleases, Vent polymerase, *Taq* polymerase, and DNA modifying enzymes from New England Biolabs, Beverly, MA, Life Technologies, Inc., or Promega, Madison, WI; and Sequenase V.2.0 and nucleotides from U. S. Biochemical Corp. The cysteine protease inhibitor E-64, MEGA-8, and all other reagents were the highest quality available and were obtained from either Sigma or Fisher.

Cell Culture—Mouse KNIH cells (Kirsten sarcoma virus-transformed NIH 3T3 fibroblasts) were a gift of Dr. C. Scher, University of Pennsylvania. Human HeLa and mouse NIH 3T3 cells were obtained from the American Type Culture Collection. Rockville, MD. Cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose, 10% fetal bovine serum, 0.005% gentamycin, 0.0025% kanamycin, and 200 mM glutamine.

Construction of Mutant cDNAs—Mutations were introduced into the propeptide of mouse procatL by the method of Kunkel *et al.* (6) using the Bio-Rad Muta-Gene kit according to the manufacturer's instructions. When possible, codons were chosen to introduce restriction sites that facilitated identification of the mutants. Mutations were confirmed by sequencing relevant regions of the modified single-stranded M13/mp19 phage DNA using Sequenase T7 DNA polymerase according to the manufacturer's instructions. The M13 replicative form was purified on a Qiagen midi-column, digested with *Bam*HI, and the resulting fragments were resolved on an agarose gel. A 1161-base pair fragment encoding mouse procatL was excised and extracted from the agarose using a 0.45 μM filter (7). The fragment was ligated into dephosphorylated, *Bam*HI-digested pSG5 vector and transformed into competent MC1061 cells (8) using standard molecular biology techniques (9). The plasmid pSK1.proCL.Q204 contains a mutation that converts the wild-type Asn^{268} to Gln^{268} (4). This plasmid and procatL/pSG5 were both digested with *Bst*XI and *Sac*II. The 630-base pair inserts were exchanged by subcloning as described above.

Construction of ProcatL-HSV Chimeric cDNA—To distinguish recombinant mouse procatL from the endogenous protein in mouse cells, a 26-amino acid C-terminal epitope tag was added to the recombinant protein. To construct the plasmid encoding this chimeric protein, the sequence encoding procatL was first subcloned into the prokaryotic expression vector pET-27b using the polymerase chain reaction (PCR). The 5′-oligonucleotide primer (CAGCAGCAGCATATGACTCCAAAATTTGATCAAACCTTT) consisted of bases encoding an *Nde*I restriction site followed by the bases encoding the first eight residues of the single chain enzyme. The 3′ primer (GTCGTCCTCGAGATTGACCACAGGATAGCT) consisted of bases encoding an *Xho*I site followed by bases complementary to the C-terminal 6 residues of procatL. The cDNA was synthesized in a 100-μl PCR reaction mix consisting of 10 ng of template (procatL/pSG5), 200 pM each primer, 2.5 mM deoxynucleotide triphosphates, 2 mM $MgSO_4$, and 3 units of Vent DNA polymerase in 1 × Vent reaction buffer. This reaction mixture was subjected to 1 cycle of 94 °C for 1.0 min, 5 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min, and 25 cycles of 94 °C for 30 s and then 72 °C for 1.5 min in a model PTC-150 thermocycler from M. J. Research, Watertown, MA. The terminal nucleotides of the PCR product were removed with *Nde*I and *Xho*I, and the product was ligated into the pET-27b vector that had been previously digested with these same enzymes.

The cDNA encoding procatL and an adjacent 78-base sequence including bases encoding the herpes simplex virus (HSV) peptide, -SQPELAPEDPED-, and six adjacent 6 histidine residues was removed from proL/pET-27b and subcloned back into the eukaryotic expression vector pSG5 using PCR. The 5′ primer (GCCAGATCTTCAGTGGTGGTGGTG) contained bases encoding a *Bgl*II site followed by bases complementary to the stop codon and those encoding 4 histidine residues, and the 3′ primer (CAGTCTTCTGTGCGTGGCCTTCCACTGGTGCCACTC) was a 36-base pair sequence homologous to the procatL proregion. This PCR product and procatL/pSG5 were both digested with *Bst*XI and *Bgl*II, and the 740-base pair inserts were exchanged. The insert was sequenced in denatured pSG5 using Sequenase T7 DNA polymerase according to the manufacturer's instructions to confirm that no mutations were introduced by PCR during subcloning.

Eucaryotic Cell Expression—Expression plasmid DNA encoding mouse procatL was introduced into HeLa cells or NIH 3T3 fibroblasts using LipofectAMINE. Plasmid DNA (6 μg) and LipofectAMINE (30 μl) were diluted individually with Opti-MEM (300 μl) and then combined and incubated for 45 min at room temperature. Cells (60-mm dishes, 80% confluent) were washed with phosphate-buffered saline (pH 7.2, PBS) and incubated with Opti-MEM (2.5 ml). The DNA-lipofectAMINE complex was then added to the cells, which were incubated for 5 h before addition of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (3 ml). On day 2, this medium was replaced with cell growth medium or serum-free medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml). On day 3, the cells were either harvested for Western blot analysis or were radiolabeled prior to immunoprecipitation of procatL. When specified, control cells were mock-transfected with LipofectAMINE alone.

Radiolabeling—ProcatL was radiolabeled by washing cell monolayers (60-mm dishes) twice with PBS, starving for 1 h in cell starvation medium (Dulbecco's modified Eagle's medium lacking methionine but containing 4.5 g/liter glucose, 8% dialyzed fetal bovine serum, 0.005% gentamycin, 0.0025% kanamycin, and 200 mM glutamine), and pulsing in cell starvation medium supplemented with [^{35}S]methionine (500 μCi/ml) for the times indicated. For experiments including chase periods, the radiolabeled medium was removed, and the cells were washed twice with PBS and incubated with cell growth medium supplemented with unlabeled methionine (5 mM) for the chase period indicated. When specified, cells were incubated with brefeldin A (20 μg/ml) for 1 h prior to the addition of medium containing the radiolabel and fresh brefeldin A. For radiolabeling with phosphate, the cells were washed, starved for phosphate, and then incubated for 4 h in phosphate-free medium containing [^{32}P]orthophosphate (500 μCi/ml).

Immunoprecipitation—Polyclonal rabbit antiserum was prepared as described previously (3) against mouse procatL expressed in bacterial cells as a fusion protein. A sample of pelleted membranes or supernatant was adjusted to contain 0.6% SDS, 50 mM Tris buffer (pH 9), 100 mM NaCl, and 2 mM EDTA, boiled for 3 min, and centrifuged in a microcentrifuge for 3–5 min. In a clean tube, the supernatant was adjusted to contain 8 mM iodoacetamide, 4% (w/v) Triton X-100, and 26 μg/ml aprotinin. Polyclonal anti-cathepsin L serum (10 μl) was added, and the sample was rotated at 4 °C for 16 h. Antigen-antibody complexes were bound to Protein A-Sepharose (40 μl of a 1:1 resin/water slurry per 10 μl of antiserum) for 3 h at room temperature. The Sepharose beads were washed four times with Tris buffer (150 mM (pH 8)) containing 150 mM NaCl, 5 mM EDTA, and 0.1% SDS. Antigen-antibody complexes were eluted from the beads by incubation at 100 °C for 3 min in PAGE sample buffer (3.3% SDS, 80 mM Tris (pH 7), 20% sucrose, 0.008% bromphenol blue, 17 mM EDTA) containing dithiothreitol (17 mM). Iodoacetamide (83 mM) was added to alkylate the thiol groups. The immunoprecipitate was analyzed by SDS-PAGE on 12% polyacrylamide gels (10) and visualized by fluorography (11). Indicated sizes of proteins were calculated based on the relative positions of commercial radiolabeled and prestained marker proteins.

EndoH Treatment—Following immunoprecipitation, samples to be digested with endo-β-*N*-acetylglucosaminidase H (EndoH) were eluted from the Protein A-Sepharose by boiling for 5 min in 50 mM Tris buffer (pH 8.0, 50 μl) containing 1% SDS and 50 mM dithiothreitol. The eluate was transferred to a clean tube. The Protein A-Sepharose was washed with 0.3 M citrate buffer (pH 5.5, 120 μl). Part (100 μl) of this wash was added to the eluate, EndoH (2 milliunits) was added, and the mixture was incubated overnight at 37 °C. Proteins were precipitated with 20% trichloroacetic acid containing yeast tRNA (25 μg/ml) for 1 h on ice and resolved by SDS-PAGE. For EndoH treatment of nonradioactive samples (in the absence of immunoprecipitation), cells were scraped into 0.5% SDS (1.0 ml/60-mm dish), heated in a boiling water bath for 5 min, and sonicated to disrupt DNA. Samples were split in half, diluted 1:10 with 50 mM NaH_2PO_4 (pH 5.5), and MEGA-8 was added to a final concentration of 0.5%. EndoH (40 milliunits) was added to one of the two duplicate samples, and both tubes were incubated at 37 °C for 16 h. The proteins were then precipitated with trichloroacetic acid as described above. For EndoH treatment of secreted proteins, cells were incubated overnight in Dulbecco's modified Eagle's medium containing insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml) (3 ml/60-mm dish). Proteins were precipitated with trichloroacetic acid, and the precipitate was resuspended and incubated with EndoH as were the cell samples. Samples treated with *N*-glycosidase F (600 milliunits) were prepared similarly, except the buffer was 20 mM NaH_2PO_4 (pH 7.2), 50 mM EDTA, 1% β-mercaptoethanol, and 0.5% MEGA-8.

Western Blot Analysis—Cells were washed twice with PBS and harvested by scraping into PAGE sample buffer (0.5 ml) (10). The lysate was sonicated for 10 s and boiled for 5 min. Secreted proteins in cell culture medium were precipitated with 20% trichloroacetic acid containing yeast tRNA (25 μg/ml) and resuspended in PAGE sample buffer containing dithiothreitol (17 mM). Solubilized proteins were resolved on 12% polyacrylamide gels and transferred to Immobilon-P (presoaked in 0.1 M CAPS (pH 11), in 10% methanol) by using a semi-dry apparatus (Integrated Separation Systems, Natick, MA) for 2 h at 1mA/cm^2. Nonspecific binding sites were blocked for 30 min with 0.5% non-fat dry milk (Carnation) in Tris buffer (10 mM, pH 7.5) containing NaCl (150

TABLE I
The LPR-inhibiting segment of mouse procathepsin L: effect of mutations on glycosylation at Asn^{268}

Mutant protein	Glycosylation at Asn^{268}	Changed residue(s)	Structural changes at pH 7
		^{33}K S T H R R L Y G^{41}	Residues of the LPR-inhibiting sequence
		+ + +	Wild-type side chain charges at pH 7
Wild type		... W K S T H R R L Y G ...	Residues of wild-type mouse procathepsin L
WKST32–35AAAA	Yes	... A A A A	Replaces four side chains at 32–35 by CH_3
HRRL36–39AAAA	Yes	... . A A A A	Replaces four side chains at 36–39 by CH_3
S34A/R37A	No	... . . A . . A	Removes oxygen at 34 and + charge at 37
S34A	No	... . . A	Removes oxygen at 34
R37A	No	... . A	Removes + charge at 37
H36A/R38A	Yes	... . A . A	Removes ring at 36 and + charge at 38
H36A	Yes	... . A	Removes imidazole ring at 36
R38A	Yes	... . A	Removes + charge at 38
L39A	No	... . A	Removes 3 carbons at 39
Y40A	Yes	... . A	Removes phenolic ring at 40
H36Y	No	... . Y	Replaces imidazole ring with phenol ring at 36
R38K	No	... . K	Replaces guandinium$^+$ with $CH_2NH_3^+$ at 38
Y40F	Yes	... . F	Removes phenolic oxygen at 40

mM) and Tween 20 (0.05%). Rabbit antiserum specific for mouse cathepsin L (diluted 1:15,000 with Tris buffer) or mouse monoclonal antiserum specific for the HSV sequence (diluted 1:20,000 with Tris buffer) was incubated with the blot for 2 h at 37 °C. The blots were washed and incubated in the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse IgG diluted 1:20,000 with Tris buffer) for 30 min at room temperature and then washed. Proteins were detected with enhanced chemiluminescence reagent (diluted 2:3 with distilled water) and exposed to film for 1–60 min.

RESULTS

LPR-inhibiting Sequence Mutants of Mouse ProcatL Are Expressed in HeLa Cells—In preparation for a study of the physiological role of the pH-dependent membrane association of procatL, the region of the procatL cDNA that encodes the LPR-inhibiting sequence (LIS) (2) was altered by site-specific mutagenesis (Table I). The various forms of mouse procatL expressed transiently in human HeLa cells were detected in cell extracts and in the cell culture medium either by immunoprecipitation after radiolabeling of the newly synthesized forms or by Western blotting of the forms present under steady-state conditions.

Expressed wild-type mouse procatL migrated as a 38-kDa protein (Fig. 1, *lanes 4–8*), similar to the endogenous proenzyme of mouse KNIH fibroblasts (Fig. 4). This mass, which was calculated using both commercial prestained and radiolabeled standards, is slightly higher than the mass of 36-kDa we reported previously based on standards radiolabeled in our own lab (12).

The endogenous human procatL of HeLa cells was distinguished from the expressed mouse proenzyme by its molecular mass. The human proenzyme migrated on SDS-PAGE as a 44-kDa protein that was barely detectable on most gels due to its weak cross-reactivity with antiserum raised against SDS-denatured mouse proenzyme (Fig. 1, *lanes 1–3, arrow*). The reason for this difference in migration of mouse and human procatL remains unclear (13).

Mutant ProcatL Is Larger than Wild-type ProcatL—The first procatL mutant studied had 4 adjacent residues in the middle of the LIS (His^{36}-Arg^{37}-Arg^{38}-Leu^{39}) replaced by 4 alanine residues. HRRL36–39AAAA lacks 2 wild-type arginine residues that are positively charged at pH 5–7 and a wild-type histidine residue that is positively charged at pH 5. When this propeptide mutant protein was expressed in HeLa cells, a 38-kDa form was detected that comigrated with wild-type mouse procatL (Fig. 1, *lane 10 versus lane 5*). In addition, a 41-kDa form was present that was absent in HeLa cells expressing the wild-type mouse protein. The mutant protein was secreted, although more slowly than the wild-type protein. After a 1-h chase, the 38-kDa form of procatL was detected in the culture medium of cells expressing the wild-type protein but was not detectable in the culture medium of cells expressing the mutant protein (Fig. 1, *lanes 7 versus lane 12*). After a 4-h chase, however, three forms of the mutant protein were present in the cell culture medium, a major 38-kDa form that comigrated with the secreted wild-type protein, a minor 41-kDa form, and a major 45-kDa form (Fig. 1, *lane 13*).

Increased Mass of the Mutant Is Due to Carbohydrate—To determine if the larger forms of this mutant protein were due to glycosylation of the polypeptide chain, they were treated with EndoH, an enzyme that removes high mannose but not complex oligosaccharide chains. EndoH treatment of the wild-type forms of procatL secreted by transfected HeLa cells decreased the mass of the 38-kDa mouse form by 2.5 kDa, as expected for removal of a single high mannose oligosaccharide chain (Fig. 2, *lane 1 versus lane 2*). Thus a complex oligosaccharide chain was not present on this protein. Similar results (not shown) were obtained for endogenous procatL secreted by mouse KNIH fibroblasts.

EndoH treatment of the 38- and 41-kDa intracellular forms of the HRRL36–39AAAA mutant produced a single protein band at 35.5 kDa, as expected for removal of all oligosaccharide chains (Fig. 2, *lane 3 versus lane 5*). Thus the difference in mass between the 38- and 41-kDa forms is due to the presence of a second EndoH-sensitive asparagine-linked high mannose oligosaccharide chain on the 41-kDa form. In contrast, EndoH treatment of the 45-kDa secreted form of this mutant protein only decreased its mass to 42.5 kDa, as expected for removal of a single high mannose oligosaccharide chain (Fig. 2, *lane 4 versus lane 6*). Thus the difference in mass between the 42.5- and 35.5-kDa forms produced by EndoH treatment is due to the presence of an EndoH-resistant complex oligosaccharide chain on the 42.5-kDa form. The 45-kDa secreted form of the HRRL36–39AAAA mutant of mouse procatL therefore contains two asparagine-linked oligosaccharide chains, one a high mannose chain and the other a complex oligosaccharide chain.

Mutants Are Glycosylated at Asn^{268}, a Normally Cryptic Site—The 38-kDa form of endogenous mouse procatL has a single high mannose oligosaccharide chain at Asn^{221} (4), but endoglycosidase treatments revealed that the 41-kDa form of the HRRL36–39AAAA mutant protein had a second high mannose oligosaccharide chain. Since these proteins have only two potential sites for the attachment of an asparagine-linked oligosaccharide chain (Asn^{221}-Asp-Thr, Asn^{268}-Cys-Ser), the sec-

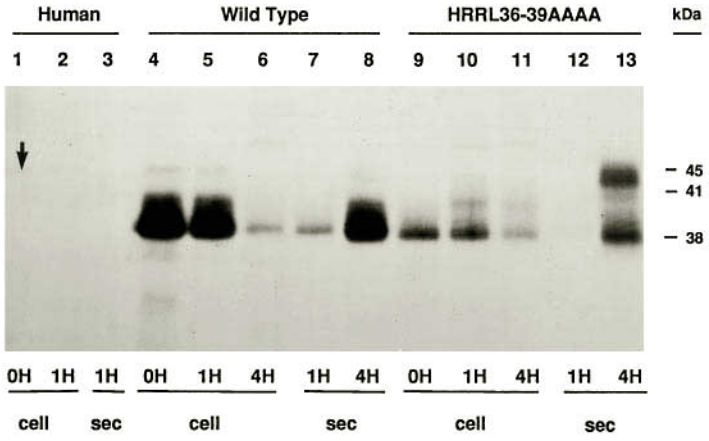

FIG. 1. **Expression of wild-type mouse procatL and the HRRL36–39AAAA mutant in HeLa cells.** Control human HeLa cells or HeLa cells transiently expressing either wild-type (*wt*) or the *HRRL36–39AAAA* mutant of mouse procatL were labeled with [^{35}S]methionine for 15 min. The radiolabel was removed and chased with unlabeled methionine for the periods specified (*H*, hours). Procathepsin L was immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) and resolved by PAGE. The *vertical arrow* (*lane 1*) points to the faint human procatL protein band. Indicated sizes of proteins were calculated based on the relative positions of commercial radiolabeled and prestained marker proteins. Much of the mouse proenzyme expressed in HeLa cells was secreted into the cell culture medium (*lane 8*), as is the endogenous enzyme synthesized by mouse KNIH fibroblasts (26).

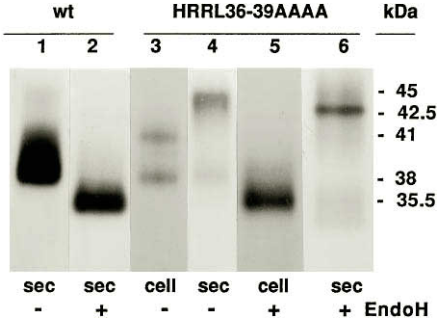

FIG. 2. **Mutant forms of procatL acquire additional carbohydrate.** HeLa cells transiently expressing either the *wild type* or the *HRRL36–39AAAA* mutant of mouse procatL were labeled with [^{35}S]methionine for 1 h. The radiolabel was removed and chased with unlabeled methionine for 4 h. Forms of procatL immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) were treated with EndoH as specified and resolved by PAGE. The figure is a composite of different exposures of a single gel.

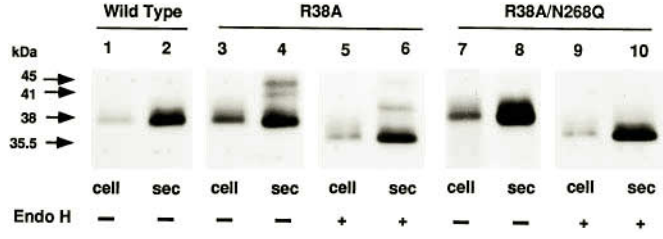

FIG. 3. **A second oligosaccharide chain is added to Asn^{268}.** HeLa cells transiently expressing *wild type,* the *R38A* mutant, or the *R38A/N268Q* mutant of mouse procatL were labeled with [^{35}S]methionine for 30 min. The radiolabel was removed and chased with unlabeled methionine for 4 h. Forms of procatL immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) were treated with EndoH as specified and resolved by PAGE.

ond oligosaccharide chain on the 41-kDa mutant proenzyme must be attached at Asn^{268}.

To confirm that the 41- and 45-kDa forms of mutant mouse procatL contained an oligosaccharide chain at Asn^{268}, this residue was replaced by mutagenesis. The N268Q mutant of mouse procatL cannot acquire a second asparagine-linked oligosaccharide chain because it contains a glutamine residue in place of Asn^{268} (4). The Arg^{38} residue of this mutant was replaced by alanine to obtain the R38A/N268Q double mutant. Like the HRRL36–39AAAA quadruple mutant, the R38A single mutant secreted the 41- and 45-kDa forms of the proenzyme (Fig. 3, *lane 4*). When the R38A/N268Q double mutant was expressed in HeLa cells, however, the 41- and 45-kDa forms were not detected either within the cells or in the cell culture medium (Fig. 3, *lanes 7* and *8*). Since they were present when Asn^{268} was present but were absent when Asn^{268} was conservatively replaced by glutamine, the 43- and 45-kDa forms of procatL must contain an oligosaccharide chain at Asn^{268}, the normally cryptic glycosylation site.

As expected, EndoH treatment of the intracellular forms of the mutants R38A and R38A/N268Q generated the 35.5-kDa form of the proenzyme, which lacks oligosaccharide (Fig. 3, *lanes 5* and *9*). EndoH treatment of the secreted forms of the R38A/N268Q mutant also generated only the 35.5-kDa form and no larger EndoH-resistant form (Fig. 3, *lane 10*). But EndoH treatment of the secreted R38A mutant also produced the larger EndoH-resistant, complex oligosaccharide form (Fig. 3, *lane 6*). Thus replacing Arg^{38} by alanine induced addition at Asn^{268} of a high mannose oligosaccharide that is later modified to complex oligosaccharide.

When wild-type procatL is synthesized in an excessive amount in transformed or transfected cells, a minor amount of the doubly glycosylated 41-kDa form of the proenzyme is detected (Fig. 1, *lanes 4, 5*, and *8*). Thus cotranslational misfolding and glycosylation at Asn^{268} might also be due in part to the limited availability of a chaperone protein needed for normal folding of wild-type procatL.

Glycosylation at Asn^{268} Does Not Correlate With Decreased Positive Charge of the Propeptide Region—Glycosylation of the HRRL36–39AAAA mutant at Asn^{268} might be due to the decreased positive charge of its propeptide region (residues 18–113) resulting from replacement of two positively charged arginine residues (Arg^{37} and Arg^{38}) by uncharged alanine residues. This possibility was explored by studying the KAKK99–102AAAA triple mutant, which has three positively charged residues (Lys^{99}, Lys^{101}, and Lys^{102}) near the C terminus of the propeptide region replaced by alanines. When this mutant was expressed in HeLa cells, the 41- and 45-kDa forms of procatL were not detected (Fig. 4, *lanes 3–7*). Like recombinant wild-type mouse procatL, the KAKK99–102AAAA mutant protein comigrated with endogenous procatL that had been immunoprecipitated from radiolabeled mouse KNIH fibroblasts (Fig. 4, *lane 1*). Also like the recombinant wild-type mouse protein (Fig. 1, *lane 7*), the KAKK99–102AAAA mutant

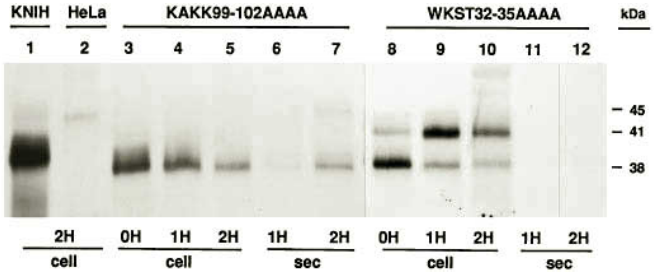

FIG. 4. **Mutations near the C-terminus of the procatL propeptide have no effect on carbohydrate modification.** Mouse KNIH cells, untransfected human HeLa cells, or HeLa cells transiently expressing either the *KAKK99–102AAAA* or the *WKST32–35AAAA* mutants of mouse procatL were labeled with [^{35}S]methionine for 30 min. The radiolabel was removed and chased with unlabeled methionine for the periods specified. Procathepsin L was immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) and resolved by PAGE. *H*, hour.

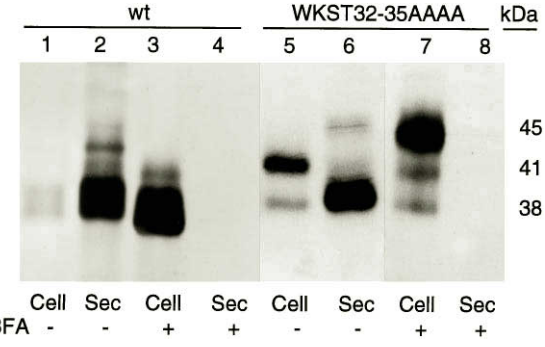

FIG. 5. **Mutant procatL modified with complex carbohydrate is only detected in cells if secretion is blocked by treatment of the cells with brefeldin A.** HeLa cells transiently expressing either wild type (*wt*) (*lanes 1–4*) or the *WKST32–35AAAA* mutant of mouse procatL (*lanes 5–8*) were labeled with [^{35}S]methionine for 30 min (*lanes 5* and *6*) or 1 h (*lanes 1–4* and *7–8*). The radiolabel was removed and chased with unlabeled methionine for 3 h (*lanes 5* and *6*) or 4 h (*lanes 1–4* and *7–8*). Procathepsin L was immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) and resolved by PAGE. When specified, the cells were treated with brefeldin A (*BFA*) for 1 h prior to and during the pulse and chase periods. Brefeldin A blocks both secretion of wild-type and mutant procatL (*lanes 4* and *8*) and conversion to mature forms in lysosomes (not shown), resulting in accumulation of the proenzyme in the cells.

was detected in the culture medium of transfected HeLa cells after a 1-h chase (Fig. 4, *lane 6*).

We also prepared WKST32–35AAAA, a propeptide mutant that lacks only one wild-type residue (Lys^{33}) that is positively charged at pH 7. When it was expressed in HeLa cells, both the 38- and 41-kDa forms of the procatL mutant were prominent within the cells. The 38-kDa forms of the KAKK99–102AAAA mutant and the WKST32–35AAAA mutant were present within the cells in comparable amounts after a 30-min pulse with [^{35}S]methionine (Fig. 4, *lane 3* versus *lane 8*). After a 2-h chase, however, the 38-kDa form was present in the culture medium of cells expressing the KAKK99–102AAAA mutant but was still absent from the culture medium of cells expressing the WKST32–35AAAA mutant (Fig. 4, *lane 7 versus lane 12*). Thus mutation of certain residues in the propeptide region of mouse procatL affects oligosaccharide modification of the protein. Merely decreasing the positive charge is not sufficient to promote glycosylation at Asn^{268}.

ProcatL Bearing Complex Oligosaccharide Is Secreted—The 45-kDa form of the mouse procatL mutants was not detected within HeLa cells during pulse-chase experiments. When secretion was blocked by incubating the transfected cells with the fungal metabolite brefeldin A, all three forms of the WKST32–35AAAA mutant were detected within the cells (Fig. 5, *lane 7*) but were absent in the cell culture medium (*lane 8*). Thus most of the 41-kDa form of this mutant, which bears high mannose oligosaccharide chains at both Asn^{221} and Asn^{268}, appears to be converted into the 45-kDa form by modification of one of its high mannose chains with complex oligosaccharide. The 45-kDa form is secreted rather than accumulating in cells.

Not All Mutants Are Glycosylated at Asn^{268}—Other residues in the N terminus of procatL were replaced by alanine, and the resulting mutants were examined for their ability to undergo glycosylation at Asn^{268} (Table I).

Unlike the single mutant R38A (Fig. 3), the single mutants S34A and R37A and the corresponding double mutant S34A/R37A were not glycosylated at Asn^{268} (Fig. 6). Substantial secreted procatL was detected in the cell culture medium 1 h after a 30-min pulse (Fig. 6, *lanes 4, 9*, and *14*), indicating that each of these three mutants moved through the secretory pathway at a normal rate. After a 3-h chase two forms of the mature enzyme were detected in cells, the 31-kDa single-chain form of cathepsin L and the 25-kDa heavy chain of the two-chain form (Fig. 6). Thus a portion of each of these mutants was targeted to lysosomes and underwent normal maturation by lysosomal proteolysis. Therefore, the hydroxyl group of Ser^{34} and the guanidinium group of Arg^{37} are not needed for transport of procatL to lysosomes.

In contrast, the single mutants H36A and R38A (Fig. 7, *lanes 1–2* and *5–6*) and the corresponding double mutant H36A/R38A (data not shown) did undergo glycosylation at Asn^{268}. When expressed in HeLa cells, each of these LPR inhibiting-sequence mutants was present within the cells in the two high mannose forms, the 38-kDa singly glycosylated form and the 41-kDa doubly glycosylated form. In addition, each of these mutants was present in the cell culture medium in the doubly glycosylated 41- and 45-kDa forms.

The two large hydrophobic residues in the LIS of mouse procathepsin L, Leu^{39} and Tyr^{40}, were also replaced by the small hydrophobic residue alanine. The Y40A mutant was glycosylated at Asn^{268} (Fig. 7, *lanes 11–12*), but the L39A mutant was not efficiently glycosylated at Asn^{268} (Fig. 7, *lanes 9–10*).

Lack of a Hydroxyl Group Can Result in Glycosylation at Asn^{268}—When His^{36}, Arg^{38}, or Tyr^{40} was replaced by alanine, the resulting mutant proenzyme was cotranslationally glycosylated at Asn^{268} (Table I and Fig. 7). Each of these residues was next individually replaced with a more conservative residue. When expressed in HeLa cells, the H36Y mutant having the hydroxyphenyl group of tyrosine in place of the imidazole group of His^{36} was not efficiently glycosylated at Asn^{268} (Fig. 7, *lanes 3–4*). Thus, unlike the small side chain of alanine, the large flat phenolic ring of tyrosine sufficiently resembles the large flat imidazole ring of histidine so that the nascent chain of the H36Y mutant folds normally during translation and only undergoes the normal glycosylation at Asn^{221}. Similarly, the R38K mutant having the positively charged methylammonium group of lysine instead of the positively charged guanidinium group of Arg^{38} was not appreciably glycosylated at Asn^{268} (Fig. 7, *lanes 7–8*).

In contrast, the Y40F mutant having the benzene ring of phenylalanine in place of the hydroxybenzene ring of tyrosine did undergo glycosylation at Asn^{268} (Fig. 7, *lanes 13–14*). This result is surprising because wild-type mouse procatL, which is not glycosylated at Asn^{268}, contains just one more atom than the Y40F proenzyme mutant, which is glycosylated at Asn^{268}. The crucial atom is the hydroxyl oxygen of Tyr^{40}. These results suggest that the hydroxyl group of Tyr^{40} participates in at least one hydrogen bond that allows the nascent chain of wild-type procathepsin L to fold during translation in a manner that prevents glycosylation at Asn^{268}.

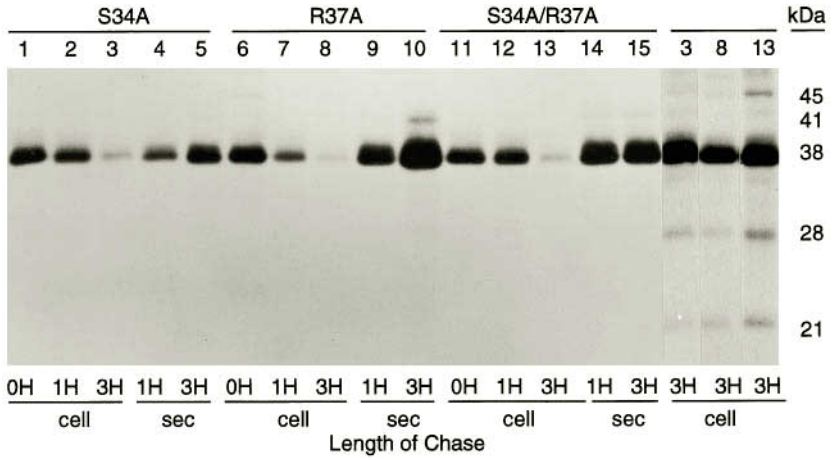

FIG. 6. **Not all propeptide mutations affect procatL folding.** HeLa cells transiently expressing the *S34A, R37A,* or *S34A/R37A* mutants of mouse procatL were labeled with [^{35}S]methionine for 30 min. The radiolabel was removed and chased with unlabeled methionine for the periods specified. *H*, hour. Procathepsin L was immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) and resolved by PAGE. A longer exposure of *lanes 3, 8*, and *13* of the same gel (*right panel*) revealed that mature forms of the three mutants are present in cells after a 3-h chase. The mature forms of mouse cathepsin L appear light relative to the proprotein bands in part because 6 of the 11 methionine residues are removed by cleavage of the propeptide.

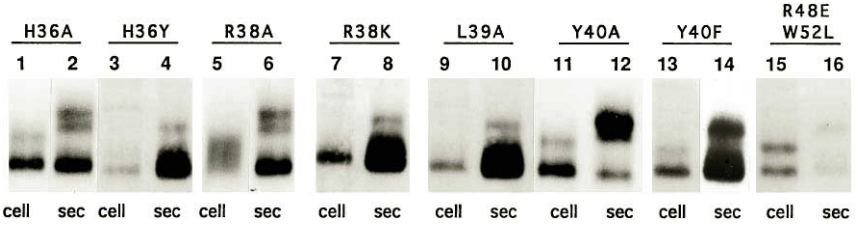

FIG. 7. **Most conservative mutations do not induce addition of carbohydrate to the cryptic Asn^{268} site.** HeLa cells transiently expressing the specified mutant forms of mouse procatL were labeled with [^{35}S]methionine for 30 min *(H36Y, R38K, L39A, Y40F,* and *R48E/W52L)* or 1 h *(H36A, R38A,* and *Y40A)*. The radiolabel was removed and chased with unlabeled methionine for 3 h following the 30-min pulse or for 4 h following the 1-h pulse. ProcatL was immunoprecipitated from the cells (*cell*) or the cell culture medium (*sec*) and resolved by PAGE. The figure is a composite of different exposures of two polyacrylamide gels.

An ERININ-Helix Mutant Is Glycosylated at Asn^{268}—The LIS (residues 33–41) precedes the ERININ region of procatL (residues 44–63), a discontinuous pattern of six residues that is conserved in several cysteine proteases (consensus: E . . . R . . . F . . . N . . . I . . . N) and folds into an α helix (14, 15). Structural changes in the ERININ helix might also change the cotranslational folding of the nascent procatL chain sufficiently to allow the normally cryptic Asn^{268} to undergo glycosylation. To test this possibility, a double mutant was prepared in an effort to retain the conformation of the ERININ α helix but change its surface charge. The positively charged Arg^{48} was replaced by a negatively charged glutamate residue, which should increase the surface charge of the ERININ α helix by +2, and the large aromatic hydrophobe Trp^{52} was replaced by the large aliphatic hydrophobe leucine, which should promote formation of an α helix. When expressed in HeLa cells, the resulting R48E/W52L double mutant was indeed glycosylated at Asn^{268} (Fig. 7, *lanes 15* and *16*). Secretion of this mutant form was reduced, however, suggesting that a significant portion of the protein may be degraded in the ER.

Mutants Can Undergo Both Glycosylation at Asn^{268} and Phosphorylation of Mannose Residues—To determine if the conformational determinant recognized by the UDP-GlcNAc: lysosomal enzyme *N*-acetylglucosamine 1-phosphotransferase (16) is preserved in the propeptide mutants, three of these mutants were expressed in HeLa cells in the presence of [^{32}P]orthophosphate, and the secreted forms of procatL were examined for the presence of the radiolabel. The 38-kDa forms of wild-type mouse procatL (Fig. 8, *lane 1*), the single mutants S34A (*lane 2*) and R38A (*lane 3*), and the double mutant R38A/N268Q (*lane 4*) were each labeled with [^{32}P]phosphate. The 41- and 45-kDa doubly glycosylated secreted forms of the R38A mutant (*lane 3*) were also phosphorylated. In each case, the radiolabel was removed by treatment with EndoH (data not shown), indicating that the [^{32}P]phosphate was attached to a high mannose oligosaccharide chain.

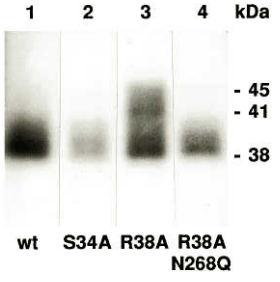

FIG. 8. **Phosphotransferase recognition of procatL is not disrupted by the addition of carbohydrate to Asn^{268}.** HeLa cells transiently expressing the specified mutant forms of mouse procatL were labeled with [^{32}P]orthophosphate for 4 h. Procathepsin L was immunoprecipitated from the cell culture medium and resolved by PAGE.

The R38A/N268Q double mutant must be folded sufficiently correctly for the phosphotransferase to recognize its single high mannose oligosaccharide chain at Asn^{221} and to attach *N*-acetylglucosamine 1-phosphate to one or more of its mannose residues. By analogy, it is likely that the R38A single mutant is also phosphorylated on its high mannose oligosaccharide chain at Asn^{221}, so that the EndoH-resistant complex oligosaccharide chain of its 45-kDa secreted form would be located at Asn^{268}.

Addition of a C-terminal Epitope Tag Induces Glycosylation at Asn^{268} and Inhibits Lysosomal Targeting—Propeptide mutants that did not acquire the second carbohydrate chain were targeted to lysosomes as revealed by the presence of mature forms of the enzyme in cells (Fig. 6). In contrast, mutants that were misfolded, as revealed by addition of the second oligosaccharide chain to at least a proportion of the expressed protein molecules, did not reach lysosomes. However, even targeting of wild-type mouse procatL was relatively inefficient in human HeLa cells, making comparison of the relative targeting efficiency of the various mutants difficult. To determine if the efficiency of lysosomal targeting of the expressed mouse mu-

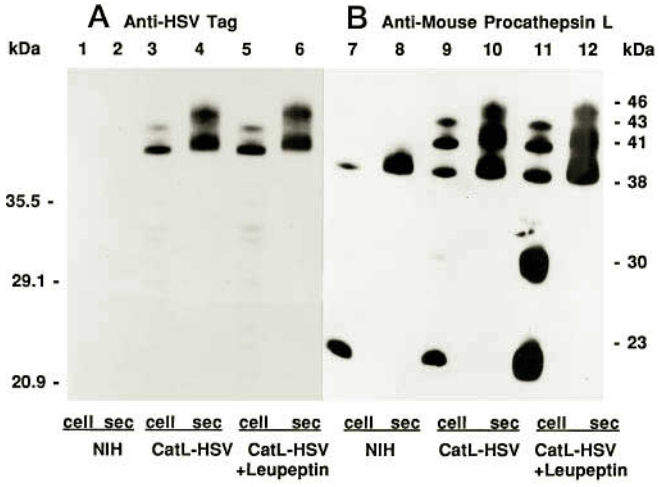

FIG. 9. **C-terminally tagged mouse procatL is not targeted to lysosomes in mouse fibroblasts.** NIH3T3 mouse fibroblasts treated with LipofectAMINE (*NIH*) or LipofectAMINE and plasmid DNA encoding HSV-tagged procatL (*CatL-HSV*) were harvested by scraping into PAGE sample buffer. When specified, cells were treated with leupeptin (50 μg/ml) for 24 h prior to harvesting. Proteins in the cell culture medium were precipitated with trichloroacetic acid and resuspended in PAGE sample buffer. Cellular (*cell*) and secreted (*sec*) proteins were resolved by PAGE and blotted to Immobilon-P. The blot was probed with antiserum specific for the HSV tag sequence (*panel A*), followed by antiserum which recognizes procatL, both the expressed and the endogenous protein (*panel B*). The positions of Bio-Rad prestained molecular mass markers (kDa) are shown on the *left*. On the *right*, the sizes of proteins, as calculated using the marker protein sizes reported by Bio-Rad, are indicated (kDa).

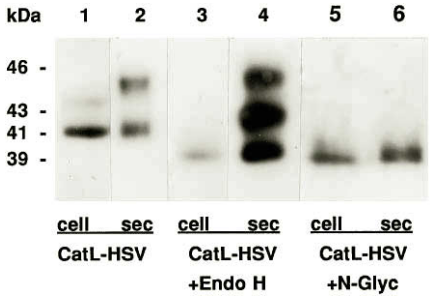

FIG. 10. **C-terminally tagged mouse procatL acquires a second oligosaccharide chain.** NIH3T3 mouse fibroblasts expressing HSV-tagged procatL (*CatL-HSV*) were harvested by scraping into PAGE sample buffer. Proteins in the cell culture medium were precipitated with trichloroacetic acid and resuspended in PAGE sample buffer. When specified, cellular and secreted proteins were treated with EndoH or *N*-glycosidase F (*N-Glyc*) prior to electrophoresis and blotting. Cellular (*cell*) and secreted (*sec*) proteins were resolved by PAGE and blotted to Immobilon-P. The blot was probed with antiserum specific for the HSV tag sequence.

tants might be greater in homologous mouse cells than in human HeLa cells, a mouse procatL mutant cDNA was constructed that encoded a 26-residue epitope tag, containing a 12-residue segment of a herpes simplex virus (HSV) protein, attached to the C terminus of the proenzyme. The epitope-tagged mouse proenzyme was expressed in mouse NIH fibroblasts, and the recombinant and endogenous forms of procatL were visualized under steady-state conditions by consecutive Western blotting with an antiserum that specifically recognizes the tag on the recombinant proenzyme, followed by an antiserum that recognizes both the recombinant and the endogenous mouse procatL.

The anti-HSV serum specific for the recombinant protein detected 41-kDa cellular and secreted forms of procatL, larger than the endogenous protein due to the presence of the extra C-terminal amino acid residues (Fig. 9, *panel A*). In addition, a 43-kDa form of tagged procatL was detected in the transfected cells (Fig. 9, *lanes 3* and *5*), and a 46-kDa form of the protein was present in the cell culture medium (Fig. 9, *lanes 4* and *6*). No mature forms of the protease were detected in cells, and no cross-reacting protein was present in the mock-transfected control cells treated with LipofectAMINE alone (Fig. 9, *lanes 1–2*). Subsequent reaction of the same blot with antiserum specific for mouse procatL detected, in addition to the above forms of expressed procatL, the endogenous forms expected (*panel B*), 38-kDa procatL and the 30-kDa single-chain and 23-kDa heavy-chain forms of the mature protease.

Failure to detect mature forms of procatL with the HSV-specific antiserum indicated that the expressed protein was not being targeted to lysosomes, even in the homologous mouse cells. To improve the chances of detecting mature forms of cathepsin L, cells were treated with leupeptin which slows conversion of the single-chain protein to the two-chain protein (Fig. 9, *panel B, lanes 7* and *9 versus lane 11*). However, the single-chain form of the epitope-tagged protein was still not detectable (Fig. 9, *panel A, lane 5*). The 25-kDa heavy chain of the two-chain form of cathepsin L cannot be detected by the HSV serum because it is derived from the N terminus of the single-chain protease and thus lacks the tag. Although it is derived from the C terminus of the single-chain protease and thus bears the HSV tag, the small light chain of the two-chain form of cathepsin L was not detected by the anti-HSV serum or the procatL antiserum because it is not retained by the 12% polyacrylamide gel used in this experiment.

The larger forms of epitope-tagged procatL appeared similar to the mutant forms of the proenzyme that acquire a second oligosaccharide chain. Treatment with EndoH converted the epitope-tagged 41- and 43-kDa cellular proteins (Fig. 10, *lane 1*) to a single 39-kDa protein (Fig. 10, *lane 3*), confirming that the 41-kDa form possessed one and the 43-kDa form possessed two high mannose oligosaccharide chains. The EndoH resistance (Fig. 10, *lane 4*) and *N*-glycosidase F sensitivity (Fig. 10, *lane 6*) of the 46-kDa secreted proenzyme indicated that this protein had been modified with complex carbohydrate. A portion of the secreted 46-kDa protein was completely EndoH-resistant, indicating both oligosaccharide chains had acquired complex carbohydrate and therefore should lack mannose 6-phosphate. The protein migrating as the middle band (Fig. 10, *lane 4*) apparently possessed one high mannose oligosaccharide chain and one oligosaccharide chain modified with EndoH-resistant complex sugars. As for several point mutations in the propeptide, the presence of the C-terminal tag sequence alters protein folding sufficiently to permit a significant portion of the expressed protein to acquire the second oligosaccharide chain at Asn^{268}. The epitope-tagged protein is secreted but is not sorted to lysosomes, which suggests that even the portion lacking the second oligosaccharide chain is misfolded.

DISCUSSION

The LPR-Inhibiting Sequence (LIS) of ProcatL—Our previous studies indicated that the mannose 6-phosphate-independent association of mouse procatL with microsomal membranes at acidic pH requires the propeptide of the proenzyme (1). A synthetic peptide containing the LIS (residues 33–41) inhibits the binding of procatL to membranes *in vitro*, suggesting that the LIS is involved in this membrane association (2). The recently published three-dimensional structure of human procatL (15) reveals that the LIS forms a solvent-exposed elbow between helices α1p and α2p, which is compatible with the LIS playing a role in the binding of procatL to the LPR. Before this structure became available, we explored the physiological function of this mannose 6-phosphate-independent membrane as-

sociation by examining the effect of changing one or more LIS residues on procatL targeting. Our studies revealed that several wild-type LIS residues are critical for normal folding and glycosylation of procatL, which has complicated a study of the role of the LIS in the cellular targeting of procatL.

Abnormal Glycosylation—We found that substantial glycosylation of the normally cryptic Asn^{268} serves as an indicator of abnormal folding of mouse procatL. Four single LIS mutants (H36A, R38A, Y40A, Y40F) underwent substantial glycosylation at Asn^{268} but five others (S34A, H36Y, R37A, R38K, L39A) did not. Alanine scanning of LIS residues 36 through 40 revealed the following pattern:

Glycosylated at Asn^{268}:	H36A	R38A		Y40A
Not glycosylated at Asn^{268}:		R37A	L39A	

This alternating glycosylation pattern suggested that these five LIS residues are folded in an extended conformation with the side chains of residues 36, 38, and 40 pointing in one direction and those of residues 37 and 39 pointing in the opposite direction. Indeed, the three-dimensional structure of human procatL (15) reveals this to be true. Specifically, the side chains of His^{36}, Arg^{38}, and Tyr^{40} all point in to the hydrophobic core of the N-terminal globular domain of the procatL propeptide, whereas the side chains of Arg^{37} and Leu^{39} both point out to solvent. Glycosylation of Asn^{268}, which lies on the surface of procatL near this domain, is blocked during the folding of wild-type mouse procatL. Replacing His^{36}, Arg^{38}, or Tyr^{40} with alanine changes the folding of this N-terminal globular domain and/or its interaction with the rest of the nascent procatL sufficiently to allow glycosylation of this normally cryptic site at Asn^{268}.

Conservatively replacing His^{36} by tyrosine or Arg^{38} by lysine maintains Asn^{268} in its normal cryptic state. But the presence of Tyr^{40} is clearly necessary to avoid cotranslational misfolding and glycosylation at Asn^{268} because it cannot be replaced by phenylalanine. This suggested that the hydroxyl group of Tyr^{40} forms a hydrogen bond that is necessary for normal folding of the nascent proenzyme. Indeed, the three-dimensional structure of human procatL (15) reveals that the hydroxyl group of Tyr^{40} forms a hydrogen bond to the carboxylate group of Asp^{82}. The absence of this single hydrogen bond evidently alters the interaction of the N-terminal globular domain with the rest of the nascent procatL sufficiently to allow glycosylation at Asn^{268}.

Phosphorylation of Mannose Residues—The enzyme that transfers phosphate to the high mannose oligosaccharide chain of wild-type procatL recognizes conformational determinants on the proenzyme (17) that are apparently preserved in representative N-terminal mutants of mouse procatL. We expected that both high mannose oligosaccharide chains of the 41-kDa form of a procatL mutant would be phosphorylated. Assay of the phosphorylation of high mannose oligosaccharide chains introduced at novel sites on cathepsin D has shown that the phosphotransferase can modify chains attached to a variety of sites on the proenzyme (18). Our results suggest, however, that only one of the two oligosaccharide chains on mutant procatL acquires phosphate. Most of the 41-kDa mutant form is transformed into the 45-kDa form having one EndoH-sensitive, high mannose oligosaccharide chain and one EndoH-resistant, complex oligosaccharide chain. Since a phosphorylated high mannose oligosaccharide chain cannot be converted into a complex oligosaccharide chain (19), only one of the two high mannose oligosaccharide chains of the 41-kDa mutant form is phosphorylated during passage through the early Golgi compartments. The normal glycosylation and mannose phosphorylation site at Asn^{221} is on the opposite side of procatL from the cryptic glycosylation site at Asn^{268} (15). Evidently the phosphotransferase cannot efficiently modify the high mannose oligosaccharide chain at Asn^{268}.

Proenzyme Secretion—The 41-kDa form of mouse procatL mutants, which has high mannose oligosaccharide chains at both Asn^{221} and Asn^{268}, is detected to a significant extent within the transfected HeLa cells and to a minor extent in the cell culture medium. The majority of the secreted mutant protein is the 45-kDa form, which is produced in the trans Golgi by conversion of the high mannose oligosaccharide at Asn^{268} of the 41-kDa form into an EndoH-resistant, complex oligosaccharide. Unless its secretion is artificially blocked by brefeldin A, the 45-kDa form is rapidly secreted and is not detected within the cells by pulse-chase assays or Western blots. Similarly, the plasma proteins α_1-proteinase inhibitor and α_2-macroglobulin are rapidly secreted from hepatocytes once they acquire complex carbohydrate (20). Thus the protein misfolding detected by addition of the second oligosaccharide chain does not significantly alter passage through the secretory pathway, but it may hinder targeting to lysosomes.

Lysosomal Targeting—Targeting of mouse procatL to lysosomes was relatively inefficient in human HeLa cells even though the proenzyme contained mannose 6-phosphate. Mature forms of the mutants were readily detected only when a mutant that did not alter protein folding, such as S34A or R37A, was expressed in a large amount. After adjusting for the number of methionine residues, 70% of newly synthesized procatL was secreted, and only about 20% was processed to mature forms. Similarly, procathepsin D targeting has been reported to be very inefficient in heterologous cells (21). This difference in lysosomal sorting between the two species is difficult to understand if only mannose 6-phosphate receptors are involved.

In an attempt to increase the targeting efficiency of mutant proteins, the mouse enzyme was expressed in homologous mouse cells instead of in heterologous human cells. To distinguish the recombinant protease from the endogenous protein, an epitope tag sequence was added to the C terminus of the recombinant protein. As seen for several of our N-terminal propeptide mutants, this modification resulted in cotranslational misfolding and addition of a second oligosaccharide chain. Similarly, we have previously observed that Asn^{268} is glycosylated when the conformation of mouse procatL is altered by insertion of a 21-residue tag sequence just before the C terminus (4). Evidently, addition of the high mannose oligosaccharide at Asn^{268} does not occur cotranslationally as the Asn^{268} site of procatL emerges from the ER membrane but after at least part of the C-terminal tag sequence has emerged from the ER membrane, when the synthesis of procatL is essentially complete. Similarly, folding of the yeast vacuolar enzyme procarboxypeptidase Y can precede glycosylation at Asn (22).

The epitope-tagged protein acquired complex carbohydrate and was secreted. Even in homologous cells it was not efficiently targeted to lysosomes, as judged by the absence of mature forms of cathepsin L. Since that part of the tagged protein which lacks the oligosaccharide at Asn^{268} also fails to target to lysosomes, it is unlikely that this second oligosaccharide chain prevents lysosomal targeting of the tagged protein. Thus either the tag itself or a conformational change it induces may prevent interaction with a sorting mediator.

The presence of the second oligosaccharide chain at Asn^{268} indicates that a mutant or tagged procatL form is misfolded. Two observations suggest that misfolding of a mutant procatL is sufficient to alter its targeting. First, the procatL mutants of procatL that were glycosylated at both Asn^{221} and Asn^{268} accumulated in the culture medium more slowly than the singly glycosylated wild-type recombinant proenzyme. After a 1-h

chase, the proportion of mutant forms in the cell culture medium was clearly less than that for the wild-type forms. But after a 4-h chase, a substantial proportion of the mutant forms was detected outside the cells. In contrast, grossly misfolded proteins are not secreted but are degraded in the ER by unknown mechanisms (5). For example, deletion of the complete propeptide region of mouse procatL results in turnover of the misfolded protein in the ER (23). Second, on cell lysis the recombinant wild-type procatL remains soluble, but the recombinant proenzyme mutants that acquire the second oligosaccharide chain aggregate to a significant extent (data not shown). This result, coupled with their slower accumulation in the cell culture medium and the addition of the second oligosaccharide chain, suggests that these mutant proteins remain misfolded to a significant degree after exit from the ER whether or not they have acquired the second carbohydrate chain. This misfolding might interfere with their ability to interact with a mediator of lysosomal targeting.

Expression of the tagged procatL might be expected to produce a dominant-negative phenotype characterized by excessive secretion of the endogenous procatL. Lysosomal targeting of endogenous procatL, however, was not altered when the misfolded tagged protein was transiently expressed. Evidently the misfolded tagged protein does not efficiently compete with the normally folded endogenous protein for interaction with the sorting mediator.

Roles of the LIS Residues—This study has produced four single LIS mutants (H36A, R38A, Y40A, and Y40F) of mouse procatL that undergo cotranslational misfolding, substantial glycosylation at Asn^{268}, and subsequent secretion. Four other propeptide mutants (WKST32–35AAAA, HRRL36–39AAAA, H36A/R38A, and R48E/W52L) also exhibit this altered phenotype. Clearly it is inappropriate to use any of these phenotypically altered mutant proteins to study the role of the LIS in the lysosomal targeting of mouse procatL.

The three-dimensional structure of human procatL (15) indicates that four LIS residues common to the human and mouse proenzymes (Lys^{33}, His^{36}, Arg^{38}, and Tyr^{40}) are structurally important because their side chains contribute to the hydrophobic core of the N-terminal globular domain. Some of them are likely to be important for the normal folding of this domain, which is probably the earliest event in the cotranslational folding of procatL. Indeed, the altered phenotype of four mouse procatL mutants (H36A, R38A, Y40A, and Y40F) is consistent with three of these LIS residues (His^{36}, Arg^{38}, and Tyr^{40}) being important for normal folding of this domain and its later interactions with the rest of the proenzyme. It is well established that a propeptide can play this chaperone role during the folding of a nascent proenzyme chain (24, 25).

In contrast, the four intervening LIS residues of mouse procatL (Ser^{34}, Thr^{35}, Arg^{37}, and Leu^{39}) have their side chains exposed to solvent near the end of the α1p helix. Some of these external LIS residues might be involved in the binding of the full-length procatL to the LPR. Also, the interaction of the LIS with the LPR may change as the protein moves from the ER, where at pH 7 the imidazole ring of His^{36} should be predominantly uncharged, to the acidic prelysosome, where at pH 5 this imidazole ring should be positively charged. Further studies of these mutants should reveal the role of these LIS residues in the lysosomal targeting of mouse procatL.

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