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 His36, Arg38, or Tyr40, the nascent chain of the mutant protein cotranslationally acquired a high mannose oligosaccharide chain at Asn268. In contrast, when alanine replaced Ser34, Arg27, or Leu39, 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 His36 or lysine replaced Arg38, Asn268 was not glycosylated. But the procathepsin L mutant having phenylalanine in place of Tyr40 was glycosylated at Asn268, which indicates that the hydrogen bond between the hydroxy1 group of Tyr40 and the carboxylate group of Asp82 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 mannosic 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) 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-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 (Asn221 and Asn268) for cotranslational attachment of an asparagine-linked high mannose oligosaccharide chain (3), but it is normally glycosylated only at Asn221 (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 also glycosylated at Asn268, 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:** [35S]methionine (Trans[35S]-label, 850–950 Ci/mmol) and [3H]labeled phosphophosphate (400–800 mCi/ml) from ICN Biomedicals, Costa Mesa, CA; Protein-A Sepharose CL-4B from Pharmacia Biotech Inc.; Dulbecco’s modified Eagle’s medium, Opti-MEM, LipofectAMINE, and fetal bovine serum from Life Technologies, Inc.; aprotinin, dithiothreitol, endo-N-acetylglucosaminidase H (EndoH), N-glycosidase F, HEPEs, pepstatin, and phe- nylmethanesulfonyl fluoride from Boehringer Mannheim; enhanced chemiluminescence Western blotting detection reagents and donkey anti-rabbit and goat anti-mouse IgG conjugated to horseradish peroxi-dase from Amersham Corp.; Immobilon-P from Millipore, Bedford, MA; brefeldin A from Epicentre Technologies, Madison, WI; Muta-Gen in vitro mutagenesis kit and prestained molecular mass marker proteins from Bio-Rad; eukaryotic expression vector pEF-27b and HSV-Tag mono-clonal antibody from Novagen, Madison, WI; plasmid purification columns from Qiagen, Chatsworth, CA; oligonucleotides from Lineberger 8808

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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 inhibition buffer, and all other reagents not otherwise noted 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 lacking methionine but containing 4.5 g/liter glucose, 8% dialyzed 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-Gen 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/mpl9 phage DNA using Sequenase T7 DNA polymerase according to the manufacturer’s instructions. The M13 replicative form was purified on a QiaGen mid-column, digested with BamHI, and the resulting fragments were resolved on an agarose gel. A 1.161-base pair fragment encoding the mutated procatL was excised from the agarose gel and ligated into the BamHI-digested plasmid pSKI. Q204 contains a mutation that converts the wild-type Asn266 to Gln266 (4). This plasmid and procatLpSSG5 were both digested with BstXI and SacII. 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′-digonucleotide primer (AGCGAGCGATATGACACCA-ATTTGATCAAACCTTT) consisted of bases encoding an NdeI restriction site followed by the bases encoding the first eight residues of the single chain enzyme. The 3′ primer (GTCGTCCTCGAGATTGACCA-GAGATTGACCA-CAGGATAGCT) consisted of bases encoding an XhoI site followed by bases complementary to the C-terminal 6 residues of procatL. The cDNA was ligated and amplified in a 100-μl PCR reaction mix consisting of 10 ng template (procatLpSSG5), 200 μM each primer, 2.5 mM deoxynucleotide triphosphates, 2 mM MgSO₄, 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 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 reaction was chilled on ice, and 1 μl of this PCR product was added to the PCR product and procatLpSSG5 were both digested with BstXI and BamHI, and the 740-base pair inserts were exchanged. The insert was sequenced in denatured pSSG5 using Sequenase T7 DNA polymerase according to the manufacturer’s instructions to confirm the mutations 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 (5 μg) and LipofectAMINE (30 μl) were mixed together 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 radio-labeled prior to immunoprecipitation of procatL. When specified, control cells were mock-transfected with LipofectAMINE alone. **Radiolabeling**—ProcatL and the radiolabeled single chain enzyme were harvested by scraping 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 [35S]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 μM) 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 [32P]orthophosphate (500 μCi/ml).

**Immunoprecipitation**—Polyconal 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 used to the addition of 0.6% SDS, 50 ml Tris buffer (pH 9), 100 ml NaCl, and 2 ml of the radiolabeled medium 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 ml of antiserum) for 3 h at room temperature. The Sepha-rose beads were washed four times with Tris buffer (150 ml (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 ml 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 radio- labeled and prestained marker proteins.

**EndoH Treatment**—Following immunoprecipitation, samples to be digested withendo-β-N-acetylglucosaminidase H (EndoH) were eluted from the Protein A-Sepharose by boiling for 5 min in 50 ml Tris buffer (pH 8) containing 1% SDS and 0.1% dithiothreitol was added, and the sample was sonicated for 10 s and boiled for 5 min. Secreted proteins in cell lysates were harvested by scraping into PAGE sample buffer (0.5 ml) containing 1% SDS and 50 mM dithiothreitol, the sample was transferred to a clean tube. The Protein A-Sepharose was washed with 0.3 ml 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 RNA (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 ml NaH₂PO₄ (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 ml NaH₂PO₄ (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 RNA (25 μg/ml) and EndoH (2 milliunits) were precipitated and resolved by SDS-PAGE. After EndoH treatment of nonradioactive samples, either before or after immunoprecipitation, cells were scraped into 0.1 mM CAPS (pH 11), in 10% methanol) by using a semi-dry apparatus (Integrated Separation Systems, Natick, MA) for 2 h at 1mA/cm². Non-specific binding sites were blocked for 30 min with 0.5% non-fat dry milk (Carnation) in Tris buffer (10 ml, pH 7.5) containing NaCl (150
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TABLE I
The LPR-inhibiting segment of mouse procathepsin L: effect of mutations on glycosylation at Asn268

| Mutant protein | Glycosylation at Asn268 | Changed residues(s) | Structural changes at pH 7 |
|----------------|------------------------|---------------------|---------------------------|
| Wild type      |                        | 7KST HRRLYG +       | Wild-type side chain charges at pH 7 |
| WKSST32–35AAAA | Yes                    | AAAAA              | Residues of wild-type mouse procathepsin L |
| HRR36–39AAAA   | Yes                    | AAAAA              | Removes four side chains at 32–35 by CH2 |
| S34A/R37A      | No                     | AAAAA              | Removes four side chains at 36–39 by CH2 |
| R37A           | No                     | AAA                | Removes oxygen at 34 and + charge at 37 |
| H36A/R38A      | Yes                    | AAAAA              | Removes oxygen at 34 |
| H36A           | Yes                    | AAAAA              | Removes + charge at 37 |
| R38A           | Yes                    | AAAAA              | Removes ring at 36 and + charge at 38 |
| L39A           | No                     | AAAAA              | Removes imidazole ring at 36 |
| Y40A           | No                     | AAAAA              | Removes + charge at 38 |
| H36Y           | No                     | AAAAA              | Removes 3 carbons at 39 |
| R35K           | No                     | AAAAA              | Removes phenolic ring at 40 |
| Y40F           | Yes                    | AAA                | Replaces imidazole ring with phenol ring at 36 |

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 HRR36–39AAAA 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 HRR36–39AAAA 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 Asn268, a Normally Cryptic Site—The 38-kDa form of endogenous mouse procatL has a single high mannose oligosaccharide chain at Asn221 (4), but endoglycosidase treatments revealed that the 41-kDa form of the HRR36–39AAAA 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 (Asn221-Asp-Thr, Asn268-Cys-Ser), the sec-

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 (His36–Arg37–Arg38–Leu39) replaced by 4 alanine residues. HRR36–39AAAA 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,
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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 [35S]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).

![Image](313x407 to 561x493)

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 [35S]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.

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

FIG. 3. A second oligosaccharide chain is added to Asn268. HeLa cells transiently expressing wild type, the R38A mutant, or the R38A/N268Q mutant of mouse procatL were labeled with [35S]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.

EndoH treatment of the secreted R38A mutant also produced the larger EndoH-resistant, complex oligosaccharide form (Fig. 3, lane 6). Thus replacing Arg38 by alanine induced addition at Asn268 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 Asn268 might also be due in part to the limited availability of a chaperone protein needed for normal folding of wild-type procatL.

Glycosylation at Asn268 Does Not Correlate With Decreased Positive Charge of the Propeptide Region—Glycosylation of the HRRL36–39AAAA mutant at Asn268 might be due to the decreased positive charge of its propeptide region (residues 18–113) resulting from replacement of two positively charged arginine residues (Arg57 and Arg59) by uncharged alanine residues. This possibility was explored by studying the KAKK99–102AAAA triple mutant, which has three positively charged residues (Lys99, Lys101, and Lys102) 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
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 (Lys33) 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 [35S]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 Asn268.

**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, lanes 5–8) or the cell culture medium (Fig. 5, lanes 7–8) 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.

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 Asn268. 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, Leu39 and Tyr40, were also replaced by the small hydrophobic residue alanine. The Y40A mutant was glycosylated at Asn268 (Fig. 7, lanes 11–12), but the L39A mutant was not efficiently glycosylated at Asn268 (Fig. 7, lanes 9–10).

**Lack of a Hydroxyl Group Can Result in Glycosylation at Asn268**—When His36, Arg38, or Tyr40 was replaced by alanine, the resulting mutant proenzyme was cotranslationally glycosylated at Asn268 (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 His36 was not efficiently glycosylated at Asn268 (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 Asn268. Similarly, the R39K mutant having the positively charged methyalammonium group of lysine instead of the positively charged guanidinium group of Arg37 was not appreciably glycosylated at Asn268 (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 Asn268 (Fig. 7, lanes 13–14). This result is surprising because wild-type mouse procatL, which is not glycosylated at Asn268, contains just one more atom than the Y40F proenzyme mutant, which is glycosylated at Asn268. The crucial atom is the hydroxyl oxygen of Tyr40. These results suggest that the hydroxyl group of Tyr40 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 Asn268.
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 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 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:lyssosomal 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 45-kDa forms of wild-type mouse procatL (Fig. 8, lane 1), the single mutants S34A (lane 2) and R37A (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.

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 resurrected 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.

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 resurrected 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.

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 44-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 antisera 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.

To improve the chances of detecting mature forms of cathepsin L, cells were treated with leupeptin which slows the 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 antisera 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^388. 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-
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...glycosylation site at Asn268 (15). Evidently the phosphotransferase cannot efficiently modify the high mannose oligosaccharide chain at Asn268.

Proenzyme Secretion—The 41-kDa form of mouse procatL mutants, which has high mannose oligosaccharide chains at both Asn221 and Asn268, 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 Asn268 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 α1-proteinase inhibitor and α2-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 Asn268 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 Asn268 does not occur cotranslationally as the Asn268 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 Asn22 (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 Asn268 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 Asn268 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 Asn221 and Asn268 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 Asn268, and subsequent secretion. Four other propeptide mutants (WKST32–35AAAA, HHRRL36–39AAAA, H36AVR38A, 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 (Lys33, His36, Arg38, and Tyr40) 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 (His36, Arg38, and Tyr40) 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 (Ser34, Thr35, Arg37, and Leu39) have their side chains exposed to solvent near the end of the α-helical region of the propeptide chain. 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 His36 should be predominately 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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