Glycosylation of procathepsin L does not account for species molecular-mass differences and is not required for proteolytic activity

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Cathepsin L is a major lysosomal cysteine proteinase in mouse and human cells. Despite similar predicted molecular masses, procathepsin L in these two species migrates on SDS/polyacrylamide gels with apparent molecular masses of 39 kDa and 42 kDa respectively. To determine if glycosylation differences account for this discrepancy, and to ascertain whether glycosylation is essential for enzymic activity, mouse and human procathepsins L were expressed at high concentrations in mouse NIH 3T3 cells or in human A431 cells after DNA-mediated transfection of cloned DNAs for these enzymes. In pulse–chase studies, human procathepsin L transfectants synthesized and secreted large amounts of enzymically active 42 kDa proenzyme and processed it into 34 kDa and 26 kDa intracellular peptides, a pattern of secretion and processing similar to that seen with endogenous or transfected mouse procathepsin L. Both translation of cloned procathepsin L cDNAs *in vitro* and Endoglycosidase H treatment of 39 kDa mouse and 42 kDa human procathepsin L resulted in non-glycosylated proteins 2 kDa lower in molecular mass than the untreated proteins for both species. This suggests that glycosylation differences are not responsible for the molecular-mass disparity between the two species. Moreover, Endoglycosidase H-treated mouse enzyme retained full proteolytic activity, indicating that glycosylation of cathepsin L is not essential for enzymic function.

INTRODUCTION

Cathepsins are lysosomal acid proteinases that are believed to play important roles in intracellular protein degradation and turnover [1], bone remodelling [2], prohormone activation [3], cancer metastasis [4] and other disease processes [5]. Although cathepsins are generally targeted to the lysosome by mannose 6-phosphate lysosomal recognition markers [6], secretory routing of several cathepsin precursors by malignantly transformed cells has been reported. These high-molecular-mass precursors include procathepsin D from oestrogen-receptor-positive breast cancer cells [7], a procathepsin from pancreatic carcinoma cells [8] and the cysteine proteinases procathepsin B from cancer ascites fluid [9] and breast tumours [10] and the major excreted protein (MEP) of transformed mouse fibroblasts [11]. MEP has been identified as the precursor to mouse cathepsin L by its enzymic properties [12] and by cDNA sequence analysis [13].

Mouse cDNA and genomic MEP/procathepsin L clones express a full-length 39 kDa protein when transfected into a variety of mammalian cell types, including human cells [13–15], and the protein is correctly processed into 29 kDa and 20 kDa forms within these cells. Acid proteinase activity increases significantly in the conditioned medium of cells transfected with cloned mouse

MEP/procathepsin L genomic DNA [14]. Transfection of a full-length human procathepsin L cDNA into mouse NIH 3T3 cells results in expression of a 42 kDa protein and two lower-molecular-mass forms that are specifically immunoprecipitated by anti-(human cathepsin L) serum [15]. Proteolytic activity in media from human procathepsin L cDNA transfectants has not previously been assessed.

Although the molecular-mass difference between the 39 kDa mouse and 42 kDa human bands detected on SDS/polyacrylamide-gel electrophoresis is 3 kDa, the predicted non-glycosylated molecular mass for both proteins is 35.8 kDa, on the basis of the deduced amino acid composition. This molecular-mass disparity suggested that mouse and human procathepsins L might be glycosylated differently. Since mouse and human procathepsins L of the appropriate molecular masses may be expressed from cDNAs transfected into either mouse or human cells, such a glycosylation difference would not be cell-type-specific.

The role of glycosylation in cathepsin L proteinase activity has not been established. Concanavalin A columns do not bind all of the applied proteinase activity of purified human, rabbit or rat liver cathepsin L [16–18]. This suggests the possibility that some of the active purified cathepsin L in these preparations is not glycosylated. Several non-cysteine proteinases clearly

Abbreviations used: MEP, major excreted protein of transformed mouse fibroblasts; Cbz-, benzyloxycarbonyl-; -NH-Mec, 4-methylcoumarin-7-ylamide.

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have enzymic function independent of glycosylation, since they retain activity when expressed as recombinant non-glycosylated enzymes in *Escherichia coli* [19–21].

Mouse and human procathepsins L were expressed in mouse and human cells by co-transfection with a multi-drug-resistance cDNA, which is selectable and amplifiable [15]. The high expression of procathepsin L in the human transfectants allowed us to examine the intracellular processing of the human procathepsin. We also evaluated the role played by glycosylation in procathepsin L proteolytic activity and in molecular-mass differences between species. We report that (1) active mouse and human procathepsins L were overexpressed by transfectants, (2) human procathepsin L secretion and processing into three intracellular forms was kinetically very similar to the secretion and processing previously described for the mouse analogue, (3) glycosylation did not account for the species differences in proenzyme molecular mass, and (4) glycosylation was not necessary for proteinase activity.

MATERIALS AND METHODS

Cell culture

Mouse NIH 3T3 cells and KNIH cells were obtained from C. Scher (University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.) and the human epidermoid carcinoma cell line A431 was subcloned from a cell line from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in monolayer at 37 °C under 5% CO₂ in air in Dulbecco's modified Eagle's Minimum Essential Medium (Biofluids, Rockville, MD, U.S.A.) containing penicillin (50 units/ml), streptomycin (50 µg/ml), L-glutamine (5 mM) and 10% (v/v) calf serum (Colorado Serum, Denver, CO, U.S.A.) for NIH 3T3 and KNIH cells or 10% (v/v) fetal bovine serum (GIBCO, Grand Island, NY, U.S.A.) for A431 cells. Cells were harvested with 0.25% trypsin (GIBCO) and 0.2 mM-EDTA in Ca²⁺/Mg²⁺-free TD buffer (0.8% NaCl/0.038% KCl/0.01% K₂HPO₄/0.1% glucose/0.3% Tris/HCl buffer, pH 7.4). Colchicine (Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted from a 10 mg/ml stock solution in dimethyl sulphoxide to appropriate concentrations in complete medium.

Plasmid construction

pHaMDR1 contains a 4380 bp *SacI*–*EcoRI* fragment of the human *MDR1* cDNA between two Harvey-murine-sarcoma-virus long terminal repeats, and its construction has been described elsewhere [15]. The cosmid pcosMEP5A is a genomic clone of the mouse procathepsin L gene and contains 5 kb of upstream sequences and 13 kb of transcribed sequences in a pSV13 vector [14]. pHaMEPh is a plasmid constructed by removing the MDR cDNA from pHaMDR1 and replacing it with the full-length human procathepsin L cDNA. First, the vector was prepared by digesting pHaMDR1 with *SacI* and *XhoI* (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) and the ends were made blunt with T4 DNA polymerase and Klenow polymerase (Bethesda Research Laboratories). In a separate reaction, the plasmid pHu16, which contains the entire human procathepsin L cDNA [22], was digested with *TaqI* (Bethesda Research Laboratories) and the ends were filled in with Klenow polymerase. The 1252 bp *TaqI* fragment (bp 141 to bp 1393 of the cDNA sequence)

was isolated and ligated with vector DNA that had been treated with calf intestinal phosphatase (Boehringer, Mannheim, Germany). A clone was isolated that contains the human cDNA in the proper orientation relative to the vector long terminal repeats.

DNA transformation and selection

NIH 3T3 and A431 cells were transfected by the calcium phosphate precipitation method [23] with pHaMDR1 + pcosMEP5A (NIH) or with pHaMDR1 + pHaMEPh (A431), and were selected with 60 ng or 6 ng of colchicine/ml respectively, as described previously [15]. To amplify the transferred DNAs, individual colonies (NIH-MEPm) or mixed populations (A431-MEPm) of cells were grown in increasing amounts of colchicine until NIH-MEPm clones were at 1 µg of colchicine/ml and A431-MEPm cells were at 400 ng of colchicine/ml. The experiments described here for NIH-MEPm cells were performed with one such selected clone. Control mouse cells selected in parallel to 1 µg of colchicine/ml contained pHaMDR1 DNA only. These cells were designated NIH-MDR.

Labelling, immunoprecipitation and electrophoresis

The 80%-confluent monolayer cultures in 100 mm culture dishes were labelled with 50 µCi of [³⁵S]methionine (Amersham, Arlington Heights, IL, U.S.A.)/ml in 5 ml of methionine-free Dulbecco's modified Eagle's Minimal Essential Medium (N.I.H. Media Unit).

Conditioned cell media were pooled and centrifuged at 300 g for 5 min to remove cell debris. To concentrate secreted proteins, (NH₄)₂SO₄ was added to the medium to 80% saturation, the solution was incubated at 4 °C for 15 min, and then the solution was centrifuged at 17000 g for 15 min. The precipitated protein pellet was dissolved in 0.5 ml of cold buffer (0.01 M-Tris/HCl buffer, pH 8.0, containing 0.01 M-NaCl) and dialysed against the same buffer overnight at 4 °C [14].

Cell lysates were obtained by treating monolayer cells with SDS buffer A (0.05 M-Tris/HCl buffer, pH 7.4, containing 0.154 M-NaCl, 0.5% Nonidet P-40 and 0.05% SDS). Lysates were clarified by centrifugation at 12000 g for 2 min.

Immunoprecipitation of [³⁵S]methionine-labelled protein was performed as described previously [24] with *Staphylococcus aureus* and either rabbit anti-(mouse MEP/procathepsin L) serum or rabbit anti-(human cathepsin L) serum. Immunoprecipitation of ³⁵S-radio-labelled protein from conditioned KNIH medium was performed with 20% Protein A–Sepharose (Pharmacia, Piscataway, NJ, U.S.A.) in 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.1% Triton X-100. Immunoprecipitated pellets were dissolved in SDS dissociation buffer [0.0625 M-Tris/HCl buffer, pH 6.8, containing 2.5% SDS, 0.0005% Bromophenol Blue, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol], boiled for 3 min and centrifuged in an Eppendorf microfuge for 3 min, and then the supernatants were electrophoresed on SDS/12%-polyacrylamide gels according to the method of Laemmli [25]. Gels were stained with Coomassie Blue, then treated with 2,5-diphenyloxazole in dimethyl sulphoxide [26] or Autofluor (National Diagnostics, Manville, NJ, U.S.A.), dried and fluorographed with preflashed X-Omat AR film (Kodak) at –70 °C. ¹⁴C-labelled molecular-mass markers were myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa),

ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa) (Amersham).

RNA synthesis and translation *in vitro*

RNA including the complete protein-coding regions for mouse procathepsin L and for human procathepsin L was synthesized from templates containing subclones of mouse [13] and human [22] cDNA clones in pGEM-3 (Promega Biotec, Madison, WI, U.S.A.). The templates were first cleaved with *Hind*III to linearize the DNA, then RNA was synthesized with the use of T7 RNA polymerase as described by the manufacturer (Promega Biotec). The RNA polymerase was allowed to react for 2 h at 37 °C before RQ1 DNAase (Promega Biotec) was added (1 unit/ μ g of DNA) for 15 min at 37 °C to degrade the DNA template. The RNA was extracted and precipitated with ethanol. Procathepsin L proteins were synthesized from this RNA by using a rabbit reticulocyte translation system *in vitro* (Bethesda Research Laboratories) in the presence of [35 S]methionine (Amersham). The reaction conditions were as recommended by the manufacturer and included 0.1 μ Ci of [35 S]methionine/ μ l and either no RNA (control) or 50 ng of RNA/ μ l per reaction. The reaction was allowed to proceed for 1 h at 30 °C, after which the RNA was degraded with RNAase A. Then 10^4 trichloroacetic acid-precipitable c.p.m. of total extract were separated by electrophoresis on an SDS/12%-polyacrylamide gel. The gel was prepared for fluorography as described above.

Endoglycosidase H treatment

Media from 80%-confluent cell cultures in a 100 mm culture dish (radiolabelled) and in six 75 cm² culture flasks were pooled and the secreted proteins were precipitated with (NH₄)₂SO₄ as described above. Concentrated proteins were then diluted with sodium phosphate buffer, pH 6.5, to a final concentration of 0.05 M and incubated for 18–20 h at 37 °C in the presence or in the absence of Endoglycosidase H from *Staphylococcus griseus* (Sigma Chemical Co.) (1 munit/1.8 μ g of protein). Samples were then tested for proteolytic activity or immunoprecipitated as described above.

Concanavalin A–Sephadex column chromatography

The 0.7 cm $\times$ 4 cm columns (Bio-Rad Laboratories, Richmond, CA, U.S.A.) were packed with 0.5 ml (5 mg) of concanavalin A–Sephadex (Pharmacia) and rinsed with 20 ml of buffer (25 mM-Hepes buffer, pH 7.5, containing 1 mM-MnCl₂, 1 mM-CaCl₂ and 1 mM-MgCl₂). Equal radioactivity amounts of Endoglycosidase H-treated and untreated concentrated KNIH ³⁵S-labelled medium samples (1 ml), which had been dialysed against the same buffer, were applied to the columns and washed with 5 ml of buffer. Column effluents and 1 ml samples not applied to the column were immunoprecipitated and either electrophoresed or assayed for proteolytic activity.

Proteinase activity

Proteinase activity of NIH-MDR, NIH-MEPm, A431 and A431-MEPH concentrated conditioned media against 5 μ M-Cbz-Phe-Arg-NH-Mec (Sigma Chemical Co.), the optimal synthetic substrate for cathepsin L, was assayed as described by Troen *et al.* [14]. Although procathepsin L is active at pH 5.5 [12], in practice we first activated the proenzyme at pH 3.0 and 37 °C for 30 s, which causes rapid conversion of procathepsin L into the

more active mature enzyme. Continuous-rate enzyme activity was then assayed at pH 5.5 and 30 °C. Leupeptin (Sigma Chemical Co.), a reversible inhibitor of cysteine proteinases, was added to the reaction mixture at a final concentration of 0.1 μ g/ml for proteinase inhibition. Protein concentration was measured by the Bio-Rad protein microassay method, with bovine serum albumin as standard. Proteinase activity was expressed as the rate of reaction during the initial linear portion of the assay in arbitrary units/s, divided by the number of cells at 80% monolayer confluence (3×10^6 NIH cells/75 cm² culture flask and 5×10^6 A431 cells/75 cm² culture flask).

Proteinase activity of concentrated KNIH-conditioned-medium immunoprecipitation complexes was assayed against 5 μ M-Cbz-Phe-Arg-NH-Mec as above, except that samples were maintained at 30 °C at pH 5.5 for 28 min. Samples were vortex-mixed every 2 min to keep the Protein A–Sephadex complexes suspended. Reactions were stopped by centrifuging immunoprecipitation complexes in a microcentrifuge and transferring reaction mixture supernatants to a fluorescence spectrophotometer for activity measurement. E-64 (Sigma Chemical Co.), an irreversible specific cysteine-proteinase inhibitor, was added at a final concentration of 2 μ M for proteinase inhibition. Activity units are expressed as pmol of substrate hydrolysed/30 min, after correction for the relative amount of procathepsin L in each sample by densitometry of electrophoresed samples (Fig. 5).

RESULTS

Expression of procathepsin L

To express mouse and human procathepsins L (MEP) at high concentrations in mouse and human cells, we co-

Fig. 1. Secretion of human procathepsin L by A431-MEPH transfectants

Cells were labelled for 19 h with [35 S]methionine, conditioned medium was pooled, and secreted proteins were concentrated as described in the Materials and methods section. Samples of concentrated proteins (8700 c.p.m. per lane) were immunoprecipitated and electrophoresed on a SDS/12%-polyacrylamide gel. Lane 1, A431; lane 2, A431-MEPH. The molecular-mass markers shown are ovalbumin (46 kDa) and carbonic anhydrase (30 kDa).

Fig. 2. Kinetics of processing and secretion of human procathepsin L.

A431-MEPH cultures in 100 mm culture dishes were pulse-labelled for 15 min with [35 S]methionine, then chased with medium containing non-labelled methionine for the indicated times. Immunoprecipitated proteins were electrophoresed on SDS/12% polyacrylamide gels. (a) 3×10^6 trichloroacetic acid precipitable c.p.m. of lysate was immunoprecipitated for each chase time, and (b) a volume of medium corresponding to the amount of lysate in (a) was immunoprecipitated for each chase time. The molecular-mass markers shown are ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). The 22 kDa band in both parts of the Figure has not been identified, but it is immunoprecipitated from control A431 medium (result not shown) and is believed to represent another secreted human protein recognized by the antibody.

transfected the cloned procathepsin L genes along with the dominant selectable human multidrug-resistance (*MDR1*) cDNA. The human *MDR1* cDNA encodes a cell-membrane efflux pump capable of conferring resistance to drugs such as colchicine, vinblastine, doxorubicin (Adriamycin) and actinomycin D [27]. Kane *et al.* [15] reported that, when NIH 3T3 cells are co-transfected with pHaMDR1 + pcosMEP5A mouse procathepsin L genomic DNA or with pHaMDR1 + pHu16 human procathepsin L cDNA, the pHaMDR1 and the procathepsin L DNA sequences are co-amplified. Both mouse and human procathepsins L are overexpressed and secreted by these cell lines.

For this work, we constructed a new human procathepsin L expression vector in an attempt to increase synthesis of the human enzyme. A cloned *TaqI* human procathepsin L cDNA fragment under control of a Harvey-murine-sarcoma-virus promoter (pHaMEPH) was co-transfected with pHaMDR1 into A431 cells. These cells were then selected with colchicine to co-amplify the transfected DNA sequences. Secretion of 42 kDa human procathepsin L by these A431-MEPH cells

(pHaMDR1 + pHaMEPH) was approx. 10-fold greater than by non-transfected A431 cells (Fig. 1).

Processing and secretion of human procathepsin L

To examine the intracellular processing and secretion of human procathepsin L, A431-MEPH cells were pulse-labelled for 15 min with [35 S]methionine, then chased with fresh medium containing unlabelled methionine for various lengths of time. The fluorograms in Fig. 2 reveal that human procathepsin L was sequentially processed into 34 kDa and 26 kDa forms within transfected A431 cells (Fig. 2a) and that approx. 50% of the 42 kDa precursor protein was secreted by these cells over 12 h (Fig. 2b). The 26 kDa intracellular form probably corresponds to the 25 kDa form of purified human liver cathepsin L previously observed on SDS/polyacrylamide-gel electrophoresis under reducing conditions [16]. The processing and secretion kinetics of transfected human procathepsin L appeared to be nearly identical with the kinetics described for endogenous mouse procathepsin L in transformed mouse KNIH cells [28]. Processing of precursor enzyme into two lower-molecular-mass intra-

Fig 3. Translation of mouse and human procathepsins L *in vitro*

^{35}S -radiolabelled proteins were synthesized *in vitro* from procathepsin L RNAs as described in the Materials and methods section. Then 10^4 trichloroacetic acid-precipitable c.p.m. of total extract per lane was separated by electrophoresis on an SDS/12%-polyacrylamide gel. Lane 1, no RNA added to translation system; lane 2, proteins made from human procathepsin L RNA; lane 3, proteins made from mouse procathepsin L RNA. Arrows indicate the mobilities of human procathepsin L (upper) and mouse procathepsin L (lower). The molecular-mass markers shown are ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). All of the bands in lanes 2 and 3 are immunoprecipitated by appropriate antisera (results not shown). The major bands seen at 40 kDa (lane 2) and 37 kDa (lane 3) are believed to be completely translated human procathepsin L and mouse procathepsin L respectively. The heterogeneous lower-molecular-mass bands in these lanes probably represent incompletely translated or prematurely terminated products.

cellular forms apparently occurs more rapidly in mouse peritoneal macrophages [29] and rat hepatocytes [30], however.

Procathepsin L molecular mass and glycosylation

To determine the molecular mass of mouse and human procathepsin L in the absence of post-translational covalent modifications, procathepsin L RNA was synthesized *in vitro* and translated *in vitro* in a rabbit reticulocyte system. As shown in Fig. 3, translation of mouse and human procathepsin L RNA *in vitro* resulted in the production of major species of unmodified mouse and human procathepsins L that migrated at 37 kDa and 40 kDa respectively on SDS/polyacrylamide-gel electrophoresis. Therefore the 3 kDa difference in molecular mass between the mouse and human procathepsins was retained when the major translation products were compared (Fig. 3). This result indicates that the molecular-mass difference was not due to differences in post-translational modification.

This result was confirmed by enzymic deglycosylation of procathepsin L expressed in mammalian cells with Endoglycosidase H, which cleaves high-mannose sugar groups from asparagine-linked glycoproteins, leaving only a single *N*-acetylglucosamine residue attached to asparagine. In Fig. 4, 2 kDa shifts in the molecular masses of radiolabelled procathepsin L from both mouse (39 kDa to 37 kDa) and human (42 kDa to 40 kDa) conditioned media after Endoglycosidase H treatment are shown.

To prove that Endoglycosidase H treatment of procathepsin L results in complete deglycosylation, concentrated radiolabelled medium from transformed mouse KNIH cells was incubated with Endoglycosidase H, then passed over a concanavalin A-Sepharose column, which binds glycoproteins. Column effluent was subjected to immunoprecipitation with specific antiserum, and immunoprecipitation complexes were either electrophoresed or assayed for enzymic activity. SDS/polyacrylamide-gel-electrophoretic analysis (Fig. 5) shows that no detectable untreated mouse procathepsin L

Fig. 4. Endoglycosidase H treatment of mouse and human procathepsins L

Cell cultures were labelled with [^{35}S]methionine for 20 h, conditioned media were pooled, and secreted proteins were concentrated as described in the Materials and methods section. Concentrated proteins were then incubated in 0.05 M-sodium phosphate buffer, pH 6.5, for 18 h at 37 °C with or without 28 munits of Endoglycosidase H. Then 6×10^5 c.p.m. of concentrated proteins per lane was immunoprecipitated and electrophoresed on an SDS/12%-polyacrylamide gel. Lane 1, NIH-MDR; lane 2, NIH-MDR + Endoglycosidase H; lane 3, NIH-MEPm; lane 4, NIH-MEPm + Endoglycosidase H; lane 5, A431-MEP; lane 6, A431-MEP + Endoglycosidase H. Arrows mark the positions of mouse and human procathepsins L untreated with Endoglycosidase H. The molecular-mass markers shown are ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). The amount of procathepsin L secreted by the control NIH-MDR cells is relatively high in this experiment compared with that previously reported for NIH cells or NIH-MDR cells [15]. NIH cells become spontaneously transformed after long periods of cultivation with a resulting increase in procathepsin L secretion [31].

Fig. 5. Concanavalin A-Sepharose chromatography of mouse procathepsin L

KNIH cells were labelled with [35 S]methionine and secreted proteins in the medium were concentrated and treated with Endoglycosidase H as described in the Materials and methods section. Then 4×10^5 c.p.m. of Endoglycosidase H-treated and untreated samples were either directly immunoprecipitated or immunoprecipitated after passage through a concanavalin A-Sepharose column, and next electrophoresed on an SDS/12%-polyacrylamide gel. Lane 1, KNIH; lane 2, KNIH, concanavalin A-Sepharose column effluent; lane 3, KNIH + Endoglycosidase H; lane 4, KNIH + Endoglycosidase H, concanavalin A-Sepharose column effluent. The positions of Endoglycosidase H-treated (arrowhead) and untreated (arrow) mouse procathepsin L are indicated. The molecular-mass markers shown are ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). Approx. 40% of the Endoglycosidase H-treated procathepsin L was not eluted from the concanavalin A-Sepharose column (compare lanes 3 and 4). It was also not eluted by washing with methyl α -mannoside (results not shown). It is therefore believed to be non-specifically bound to the column.

passed through the column, whereas over 60% of the treated proenzyme was recovered from the column effluent. Mouse procathepsin L in the Endoglycosidase H-treated column effluent migrated at 37 kDa, indicating that the 2 kDa decrease in molecular mass observed after Endoglycosidase H treatment in the previous experiment (Fig. 4) reflects genuine deglycosylation. Together, these experiments confirm that the 3 kDa molecular-mass difference between the human and mouse proenzymes shown by SDS/polyacrylamide-gel-electrophoretic analysis is not caused by differential glycosylation or other post-translational modifications.

Enzymic activity and glycosylation

Conditioned media from 35 S-radiolabelled NIH-MEPm and NIH-MDR (control) cells were assayed for cathepsin L activity after incubation at 37 °C with or without Endoglycosidase H. Activity was assayed with the synthetic substrate Cbz-Phe-Arg-NH-Mec and the

Table 1. Activity of mouse and human cathepsins L

Medium from 80%-confluent cell cultures conditioned for 20 h was pooled, concentrated and assayed for activity as described in the Materials and methods section.

Cell type	Endoglycosidase H	Leupeptin	Activity (units/s per 10^6 cells)
NIH-MDR	—	—	5.8
NIH-MDR	+	—	5.8
NIH-MDR	—	+	1.0
NIH-MEPm	—	—	16.7
NIH-MEPm	+	—	19.0
NIH-MEPm	—	+	4.4
A431	—	—	1.7
A431-MEPH	—	—	25.8
A431-MEPH	—	+	8.0

proteinase inhibitor leupeptin. As shown in Table 1, the leupeptin-inhibitable specific activity of the NIH-MEPm medium was 2.2 times greater than that of the NIH-MDR (control) medium. This paralleled the 2–3-fold greater secretion of immunoprecipitable mouse procathepsin L by these particular NIH-MEPm cells (Fig. 4). Although neither the substrate nor the inhibitor in this experiment is absolutely specific for cathepsin L, the higher proteinase activity in medium from procathepsin L transfectant cells that oversecrete immunoprecipitable procathepsin L relative to control cells can be attributed to expression of the transfected procathepsin L. Endoglycosidase H treatment did not result in diminished proteolytic activity in medium from either cell line (Table 1), despite the fact that the sugar had been removed by this treatment (Fig. 4). Likewise, Endoglycosidase H treatment did not result in diminished cathepsin L activity in medium from KNIH cells, but the 2 kDa molecular-mass decrease was again seen (Fig. 5). Pellets immunoprecipitated by specific anti-(mouse cathepsin L) serum from treated and untreated concentrated medium samples were assayed for proteinase activity with Cbz-Phe-Arg-NH-Mec and the specific cysteine-proteinase inhibitor E-64. No difference in specific activity (as defined in the Materials and methods section) was observed between the Endoglycosidase H-treated samples (60 units) and untreated samples (54 units), and the treated concanavalin A-Sepharose column effluent samples retained 80% of their pre-column activity (48 units). Activity in all samples was 95% inhibited by E-64. These data suggest that glycosylation of mouse cathepsin L is not essential for enzymic function.

Table 1 also illustrates that conditioned medium from A431-MEPH transfectants contained 15-fold higher leupeptin-inhibitable proteinase activity than did A431 control medium. This proteolytic activity and the increased concentrations in A431-MEPH medium of a 42 kDa protein precipitated by specific anti-(human cathepsin L) serum (Fig. 1) indicate that the 1252 bp *TaqI* cDNA fragment in pHAMEPH encodes full-length functional human procathepsin L, as predicted by the DNA sequence.

DISCUSSION

Both human and mouse cathepsins L contain asparagine-linked high-mannose sugars. To date the only significant function assigned to the *N*-linked glycosylation of cathepsin L is that of targeting the enzyme to the lysosomes of either the host cell [32] or of other cells that display mannose 6-phosphate surface receptors [32,33]. In the present paper we have shown that glycosylation has no apparent effect on enzymic activity of secreted mouse procathepsin L. We have also recently expressed purified recombinant human procathepsin L from a cloned cDNA fragment in *Escherichia coli* (S. M. Smith & M. M. Gottesman, unpublished work). This recombinant enzyme is proteolytically active with a similar specific activity to that of purified human liver cathepsin L [16], confirming that glycosylation of human cathepsin L is not required for proteolytic activity.

The deduced amino acid sequences of mouse and human procathepsins L [22] indicate the presence of two and one potential asparagine-linked glycosylation sites respectively. Judged by the same 2 kDa decrease in molecular mass observed for both mouse and human procathepsins L after enzymic deglycosylation or translation *in vitro*, both enzymes carry an equivalent number of sugar residues. This suggests the possibility that only one of two potential glycosylation sites on mouse procathepsin L is utilized.

The predicted molecular masses of mouse and human procathepsins L are the same, yet the molecular masses observed on SDS/polyacrylamide-gel electrophoresis differ by 3 kDa, regardless of whether expression is in mouse or human cells. Moreover, this 3 kDa difference persists in the non-glycosylated forms of the two proteins, indicating that glycosylation cannot account for the difference in SDS/polyacrylamide-gel-electrophoretic migration. The most plausible explanation for this phenomenon is that different charges on the two proteins result in different gel mobilities. On the basis of the deduced amino acid compositions of the precursor and processed cathepsin L peptides, all three forms of the mouse enzyme are more basic than their human enzyme counterparts, each mouse form having seven to ten fewer net acidic groups. Since basic proteins tend to migrate relatively faster on SDS/polyacrylamide-gel electrophoresis than do acidic proteins [34], presumably owing to increased SDS binding, the faster mobility of each of the mouse cathepsin L forms (39 kDa, 29 kDa and 20 kDa) relative to their human cathepsin L counterparts (42 kDa, 34 kDa and 26 kDa) is consistent with the more basic isoelectric point of the mouse enzyme [24].

These studies establish that it is possible to express human and mouse procathepsins L at high concentrations in cultured cells after DNA-mediated transfection of appropriate cloned sequences. The secretion and processing of mouse and human procathepsins L are virtually identical, and the species differences in molecular mass are not due to differences in glycosylation. Glycosylation is not essential for enzymic activity of cathepsin L from either species.

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