Biosynthesis and Processing of Cathepsin G and Neutrophil Elastase in the Leukemic Myeloid Cell Line U-937

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The processing of the neutral proteases cathepsin G and neutrophil elastase, normally synthesized in myeloid precursor cells and stored in azurophil granules, were investigated by biosynthetic labeling with \(^{14}\)C-leucine of the monoclastic cell line U-937. The proteases were precipitated with specific antibodies and the immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography. The transfer to lysosomes of newly synthesized proteases was demonstrated in pulse-chase labeling experiments followed by centrifugation of cell homogenates in a Percoll gradient. The presence of a closely spaced polypeptide band-doublet at intermediate gradient density suggested cleavage of the specific aminoterminal pro dipeptide extension before storage in lysosomes. The molecular heterogeneity observed for cathepsin G and neutrophil elastase seemed to be due to modifications occurring after sorting into lysosomes, most likely because of C-terminal processing. Modifications of the secreted enzymes were not detectable by SDS-PAGE. In contrast to other lysosomal enzymes, no phosphorylation was demonstrated. Newly synthesized cathepsin G and neutrophil elastase rapidly became resistant to endoglycosidase H, indicating transport through the medial and trans cisternae of the Golgi complex and conversion to "complex" oligosaccharide side chains. This conversion was inhibited by an agent swainsonine, but translocation from the Golgi complex and secretion were unaffected. The processing described may play a role in activation of the proteases.

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Bio-Rad (Richmond, CA); and Amplify solution for fluorography was from Amersham (Amersham, England).

Antiserum to cathepsin G and neutrophil elastase were obtained by immunization of rabbits as previously described.12,11

Cell culture. U-937 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of air/CO₂ (19:1). Two subclones, U-937-3 and U-937-4 (kindly provided by Kenneth Nilsson, Uppsala, Sweden), were used. Exponentially growing cells were used in all studies.

Labeling with ³⁵S-leucine. U-937 cells were starved in leucine-free minimum essential medium (Eagle) supplemented with 15% dialyzed FBS at 37°C for 60 minutes to allow depletion of the intracellular leucine pool. The labeling medium was also leucine-free with 15% dialyzed FBS. The cells, 5 x 10⁶/mL, were incubated for 30 minutes in 20 mL with 50 µCi/mL of ³⁵S-leucine. For chase of the label, the cells were resuspended in RPMI 1640 medium with 10% FBS at a density of 2.5 x 10⁶ cells/mL and incubated for various time periods when necessary. Swainsonine was added at a final concentration of 100 µmol/L. Swainsonine, added to the cell cultures 90 minutes before addition of the label, was also present during pulse labeling and chase of the label.

In phosphorylation experiments, 5 x 10⁶/mL cells were incubated in phosphate-free minimum essential medium (Eagle) supplemented with 10% dialyzed FBS and carrier-free ortho³²P-phosphate (250 µCi/mL).

Subcellular fractionation. Homogenization of cells (10⁶ per milliliter) was performed in 0.34 mol/L-sucrose/5 mmol/L HEPES (pH 7.3)/0.5 mmol/L EDTA with 40 to 60 strokes using a Dounce glass homogenizer ( Kontes, Vineland, NJ). PMSF, 1 mmol/L, was added in all steps. The homogenate was diluted with the same solution, and unbroken cells and nuclei were pelleted by centrifugation at 700g for 10 minutes. For density gradient separations, 6 mL of 20% Percoll in HEPES/sucrose adjusted to give a final concentration of 15 mmol/L HEPES pH 7.4 and 0.25 mol/L sucrose was layered on top of a 1-mL cushion of saturated sucrose. The 700g supernatant of the cell homogenate (2 mL) was layered on top of the Percoll. Centrifugation was performed at 32,000g for 60 minutes in a Sorvall RC-5B centrifuge (Du Pont, Wilmington, DE) using the SE-12 rotor. Fractions were collected from the bottom with a peristaltic pump. The fraction volume was 0.8 mL, unless otherwise stated, except for the last fraction (no. 9) in which the cytosol was collected. Refractory indices were determined by use of a refractometer to calculate the density.

In some cases a modified Percoll centrifugation was used. The postnuclear supernatant was centrifuged on a cushion of 90% Percoll in HEPES/sucrose adjusted to a final pH of 7.4 and 0.25 mol/L sucrose. A lysosome (granule) fraction was collected at the interphase by centrifugation at 6,000g for 15 minutes. The supernatant was also recovered and designated post-lysosomal supernatant.

To identify the subcellular sites involved in protein synthesis, cells (10 mL, 3 x 10⁶/mL) were incubated at 37°C for 10 minutes with (³⁵S)leucine (50 µCi/mL) followed by addition of cycloheximide (100 µg/mL). Cycloheximide was also added to the homogenization medium. Homogenates of cells labeled with (³⁵S)leucine were subjected to Percoll density gradient centrifugation and 15 fractions were collected. Aliquots of the Percoll fractions were treated with trichloroacetic acid (TCA) precipitated and radioactivity measured in a scintillation counter. Galectosyl transferase activity of fractions was assayed as described above. Distribution of lysosomes was monitored by assaying for β-hexosaminidase activity as previously described.10

Immunoprecipitation of cathepsin G and neutrophil elastase. For the immunoprecipitation, cellular organelles were solubilized in 0.3% cetyl trimethylammonium-bromide (CTAB)/0.15 mol/L NaCl/1 mmol/L PMSF/5 mmol/L HEPES, pH 7.4 (lysosome buffer). Percoll density-gradient fractions were diluted with ½ vol of fivefold concentrated lysosome buffer and 3 vol of H₂O. All lysates were kept on ice for 1 hour and then clarified by centrifugation at 4°C at 32,000g for 2 hours. Supernatants were stored frozen until used for immunoprecipitation.

Lysates and cell-conditioned media (up to 10 mL) were mixed with 15 µL antiserum and left on ice overnight. Forty microliters of Protein A bound to Sepharose (200 mg/mL lysosome buffer) (Pharmacia, Uppsala, Sweden) was added for collection of the immune complexes by rotation at 4°C for 60 minutes. For immunoprecipitation of the extracts of Percoll density gradient fractions, the appropriate amount of antiserum was bound to Protein A-Sepharose before addition of the extract. Thereafter the mixture was rotated overnight. The Protein A-Sepharose samples were washed five times in lysine buffer. The pellet was resuspended in 50 µL of water plus 15 µL of electrophoresis sample buffer (0.4 mol/L Tris/HCl[pH 6.8]/50% [vol/vol] glycerol/10%/wt/vol)SDS/5%/wt/vol) mercaptoethanol), placed in boiling water for 5 minutes, and subjected to electrophoretic analyses on 5% to 20% linear acrylamide gradient gels followed by fluorography and exposure of the gels to x-ray films.23 The molecular weight markers included phosphorylase b (94 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (20.1 Kd), and α-lactalbumin (14.4 Kd).

Digestion with endoglycosidase H. Immunoprecipitates collected on Protein A-Sepharose as described above were suspended in 10 mL 0.1 mol/L Tris buffer, pH 8.0, containing 1% sodium dodecyl sulfate (SDS) (wt/vol) and 1.5% (wt/vol) β-mercaptoethanol, and placed in boiling water for 4 minutes. After centrifugation, the supernatant was removed, diluted with 9 vol 0.15 mol/L sodium citrate, pH 5.5, and divided in two equal volumes. One volume received 0.04 U of endoglycosidase-H (endo-H); the second volume received no additives. After incubation at 37°C for 15 hours, each sample received 30% (wt/vol) trichloroacetic acid. The precipitate formed was washed three times with ice-cold acetone, dissolved in electrophoresis sample buffer, and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) analyses as described above.

RESULTS

Biosynthesis of cathepsin G and elastase in subclones of U-937. Two subclones of the monoblastic cell line U-937 (subclones U-937-3 and U-937-4) were used to investigate the biosynthesis and processing of cathepsin G and elastase. Figure 1 shows the results of biosynthesis of cathepsin G in U-937-3 cells. The 32-Kd band corresponds to the newly synthesized form of cathepsin G. On chase of the label for 90 minutes, most of the 32-Kd peptide was observed in the medium, whereas some was transferred to the lysosomal fraction where processing to lower molecular weight forms took place with time as described below. The specificity is demonstrated by the absence of the 32-Kd peptide in samples of lysosomes and incubation medium treated with preimmune serum (Fig 1). However, because of the heavy background no specific product could be demonstrated in the post-lysosomal supernatant fraction. Specificity tests using anti-cathepsin G blocked with cathepsin G were not useful because the addition of cathepsin G resulted in the precipitation of most of the protein content of the antiserum. Newly synthesized mol wt 33 Kd elastase was also demonstrated in U-937-3 and seemed to be secreted completely, and none was...
observed in the lysosomal fraction (data not shown). U-937-4 produced less cathepsin G but more elastase than U-937-3. On the basis of these results we chose to investigate the processing of cathepsin G in U-937-3 and the processing of elastase in U-937-4 cells.

Subcellular processing of newly synthesized cathepsin G and elastase. A self-generating Percoll density gradient was used to separate subcellular organelles (Fig 2). The lysosome marker β-hexosaminidase was detected at the bottom of the gradient (dense lysosomes), but a peak of β-hexosaminidase was also observed in the upper part of the gradient ("light" lysosomes). Golgi elements identified by the distribution of galactosyl transferase and subcellular sites for protein synthesis identified by a short-time incubation with (3H)leucine were found in the upper part of the gradient. A clear separation was achieved between dense lysosomes and other organelles. Golgi elements identified by the distribution of galactosyl transferase showed a somewhat higher density than subcellular sites for protein synthesis and a somewhat lower density than "light" lysosomes.

After 25 minutes of labeling, 14C-labeled 32-Kd cathepsin G was detected in all gradient fractions except those containing dense lysosomes (Fig 3A). A distinct band doublet was seen on the gel in samples derived from fractions with intermediate density, although it was difficult to demonstrate this in Fig 3 (fractions 5 through 7 of Fig 3A) because the two bands are located very close to each other. This finding suggests a rapid processing step that takes place in subcellular organelles with an intermediate density. Chase of the label for 90 minutes showed that the newly synthesized cathepsin G was transferred to fractions with a high density (Fig 3B). In addition to the 32-Kd chain, a processed 30-Kd chain was also present in gradient fractions with intermediate and high density. After 6 hours of chase a further processed form of 28 Kd was also visible whereas the 32-Kd polypeptide was no longer present (Fig 3C). Results from longer periods of chase demonstrated that the mol wt 28-Kd polypeptide was the predominant cathepsin G form (data not shown).

A similar processing of elastase was also observed (Fig 4).

Fig 2. Profile of biochemical markers in fractions from Percoll density gradient centrifugation of postnuclear supernatant of U-937-3 cells. Postnuclear supernatant was centrifuged on 20% Percoll for 60 minutes at 32,000g. Fourteen 0.5-ml fractions were recovered; fraction no. 16 contains all cytosol (2 mL) that remains on top of the gradient. The distribution of β-hexosaminidase (○-○) indicates the location of lysosomal-like organelles, and the distribution of galactosyl transferase (□-□) indicates the location of Golgi elements. The distribution of (14C)leucine-labeled macromolecules after 10 minutes of pulse labeling (■-■) identifies subcellular sites for newly synthesized protein. All activities are given as percentage of total.
Effects of swainsonine on processing. Figures 5 and 6 show results of pulse-chase experiments and demonstrate the processing of newly synthesized and secreted cathepsin G and elastase in absence and presence of swainsonine. In the absence of swainsonine, cathepsin G rapidly became resistant to digestion with endo-H. After a pulse-labeling period of 25 minutes most of the polypeptide was resistant to endo-H, indicating conversion of a “high mannose” to a “complex” sugar configuration of N-linked oligosaccharides known to occur in the medial cisternae of the Golgi apparatus. Secreted cathepsin G was not altered by endo-H. Further, swainsonine did not appear to affect the size of newly synthesized cathepsin G in a visible way as judged by SDS-PAGE. The effect of swainsonine occurred subsequent to precursor synthesis. The forms produced in the presence of swainsonine were clearly distinguished from the forms produced by control cells as judged by their susceptibility to endo-H (Fig 5). Cathepsin G synthesized in the

Fig 3. Pulse-chase labeling with (14C)leucine of cathepsin G in U-937-3 cells with distribution of labeled cathepsin G in subcellular fractions. Cells were labeled for 25 minutes (A), and the label was chased for 90 minutes (B) and 6 hours (C). The postnuclear supernatants were centrifuged on 20% Percoll for separation of subcellular organelles, and nine fractions were collected and analyzed. Extraction, immunoprecipitation with anti-cathepsin G serum, SDS-PAGE, and fluorography were performed as described in the text. The fluorogram of (A) was exposed for 4 to 5 days while the fluorograms of (B) and (C) were exposed for 3 weeks. The position of newly synthesized mol wt 32-Kd cathepsin G is indicated with an arrow to the right: broken arrows indicate the mol wt 30- and mol wt 28-Kd processing forms.

The longer the period of chase the more labeled elastase was present in the gradient fractions containing dense lysosomes. The mature elastase product appeared to be a 29-Kd polypeptide.

Fig 4. Pulse-chase labeling with (14C)leucine of elastase in U-937-4 cells with distribution of labeled elastase in subcellular fractions. Cells were labeled for 25 minutes (A), and the label was chased for 3 hours (B). The postnuclear supernatants were centrifuged on 20% Percoll for separation of subcellular organelles, and nine fractions were collected and analyzed. Extraction, immunoprecipitation with anti-elastase serum, SDS-PAGE, and fluorography were performed as described in the text. The fluorogram of (A) was exposed for 5 days while that of (B) was exposed for 3 weeks. The position of newly synthesized mol wt 32-Kd elastase is indicated with an arrow to the right: a broken arrow indicates a mol wt 29-Kd processing form.
presence of swainsonine was sensitive to endo-H. Further swainsonine blocked the normal carbohydrate processing of cathepsin G from endo-H–sensitive states to endo-H–resistant states. Finally, cathepsin G secreted by swainsonine-treated cells was partially susceptible to endo-H, indicating that formation of complex oligosaccharide side chains is not necessary for secretion of this protein. The presence of 20-Kd and 10-Kd forms in the cell-conditioned medium is probably the result of degradation of cathepsin G.

Figure 6 shows that the effect of swainsonine on the processing of neutrophil elastase was similar to that on cathepsin G. Newly formed elastase remained susceptible to endo-H, as observed after pulse labeling of cells for 25 minutes, but after chase of the label the secreted elastase was resistant. In contrast, swainsonine-treated cells secreted elastase that was susceptible to endo-H.

Swainsonine can be used in combination with endo-H to estimate the number of oligosaccharide side chains of the polypeptide. The presence of three bands (original and two produced after treatment with endo-H) corresponding to cell-secreted elastase indicated the presence of two oligosaccharide side chains per molecule of elastase. This result is consistent with the presence of two N-glycosylation sites on elastase as deduced by characterization of the elastase cDNA.15 Similarly, swainsonine-treated cells secrete cathepsin G, which on digestion with endo-H yields two bands (one original and one produced by endo-H) consistent with the finding that the cathepsin G gene codes for a protein with one N-linked glycosylation acceptor site.16

Lack of phosphorylation. Incubation of U-937 cells with 32P-phosphate for 60 minutes repeated twice did not result in detectable phosphorylation of cathepsin G and elastase (data not shown).

DISCUSSION

The results presented in this report elucidate some steps involved in the biosynthesis and processing of cathepsin G and neutrophil elastase. These neutral proteases are present both in neutrophils and monocytes, but neutrophils as well as resting and activated monocytes do not contain detectable neutrophil elastase messenger RNA (mRNA) transcripts.23 In contrast, bone marrow precursor cells contained mRNA transcripts consistent with the packaging of the neutral proteases into azurophil granules.25 Results from investigation of the biosynthesis of cathepsin G and elastase in the monoblastic cell line U-937 provide information about the processing of these proteins, and the results are regarded as relevant also for the neutrophil series of myeloid cells.

The N-terminal of cathepsin G contains an 18 amino acid signal peptide.16 Cleavage of the signal peptide generates a zymogen molecule ending at the “pro,” dipeptide extension Gly-Glu.16 Similarly, analysis of the N-terminal peptide sequence of neutrophil elastase suggests that it contains a 27-residue “pre” signal peptide followed by a “proN,”
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Pulse Chase

Post-Granules Granules Medium
Endo H + - -
Swain. - - -

Mr x 10^3
94
67
43
30
20
14

Fig 6. Effect of swainsonin (Swain.) on the biosynthesis and secretion of elastase from U-937-4 cells. Cells were labeled for 25 minutes with (14C)leucine (pulse) and chased for 90 minutes (chase) in nonradioactive medium. Cell homogenates were separated with the modified Percoll centrifugation method to obtain a lysosome fraction (granules) and a postlysosomal supernatant fraction (post-granules). All samples including the medium obtained after chase were immunoprecipitated with anti-elastase. Pluses and minuses in the row marked swain indicate the presence or absence of swainsonine during the pulse-chase period. Pluses in the row marked Endo H indicate treatment with endo-H before electrophoresis. The position of newly synthesized mol wt 33-Kd elastase is indicated with an arrow to the right; broken arrows indicate position of products obtained on treatment with endo-H.

dipeptide. There is a remarkable similarity between serine proteases derived from bone marrow cells such as cathepsin G, neutrophil elastase, and mast cell protease as they exhibit an amino terminal propeptide that has the sequence X-Glu.' The neutrophil elastase and cathepsin G genes are only expressed in bone marrow myeloid progenitor cells. The pro, peptide of pancreatic cell serine proteases consists of 6 to 20 amino acid residues and ends in Arg or Lys. Our observation that a cathepsin G protein doublet was present in the subcellular fractions with intermediate density on Percoll gradient suggests that cleavage of the propeptide took place before storage in lysosomes. We assume that neutrophil elastase was processed similarly, although a doublet of newly synthesized peptide was not visible on SDS-PAGE analysis. The results did not allow us to determine whether cleavage of the propeptide took place before the point where the secretory and storage pathways diverge; therefore, it is not known whether the secreted product was activated. Cathepsin G and neutrophil elastase were subjected to further modification subsequent to or during storage, but such modification could not be detected in the secreted product by SDS-PAGE analysis.

Neutrophil elastase and cathepsin G are formed in the progenitor cells of the bone marrow and stored in azurophil granules. These granules resemble lysosomes, and it was possible to demonstrate storage in lysosomes of the U-937 subclones. On the other hand, the targeting to lysosomes did not seem to be similar to lysosomal enzymes in general. Such enzymes require phosphorylation of mannose residues of oligosaccharide side chains. This phosphorylation occurs in the cis part of the Golgi complex and is followed by attachment to a receptor for mannose-6-phosphate, which directs transfer to lysosomes. Cathepsin G and neutrophil elastase were not found to be phosphorylated. Therefore, an alternative as yet unknown mechanism(s) is involved in transport of these proteins. Another constituent of azurophil granules of myeloid cells, MPO, is phosphorylated on oligosaccharide side chains, and in contrast cathepsin G and neutrophil elastase remains susceptible to endo-H. However, it is not likely that phosphorylation of MPO is important for directing intracellular transport in bone marrow cells; therefore, the targeting mechanism for MPO also seems to be unknown. It is possible that the enzymes destined for azurophil granules may have similar sorting mechanisms even if they differ in the oligosaccharide side chain processing.

Initially we purified four cationic proteins, with mol wts of 25.5 to 28.5 Kd, which now are designated cathepsin G. Similarly, neutrophil elastase was found to consist of three components. The present results suggest that the heterogeneity previously observed is due to modifications that take place after sorting into lysosomes. The modifications could result by deglycosylation and/or C-terminal processing. Neutrophil elastase contains a 20 amino acid C-terminal
“pro” peptide that is not found in the mature protein. Also, cathepsin G has a predicted pro peptide. Therefore, the time-dependent decrease in the mol wt of neutrophil elastase and cathepsin G of lysosomes may be due to C-terminal processing of these proteins. In contrast, secreted elastase and cathepsin G did not appear to be modified, indicating that they represent proenzymes that are not activated before secretion. This result is consistent with the presence of minimal elastase activity in medium conditioned by U-937 cells.

Newly synthesized cathepsin G and neutrophil elastase rapidly acquire resistance to endo-H, indicating formation of complex oligosaccharides in the medial cisternae of the Golgi complex. Presence of swainsonine during processing resulted in aberrant products; both cathepsin G and elastase exhibited an increased sensitivity to endo-H that resulted in molecules with decreased size on SDS gels. On the basis of these results we conclude that swainsonine prevents processing of high mannose side chains to endo-H–resistant (complex) structures. Although abnormal carbohydrate processing occurred in the presence of swainsonine, the translocation of the enzymes from the Golgi apparatus was not affected. Thus, secretion of these enzymes is independent of swainsonine-induced modifications of the N-linked oligosaccharide chains.

In conclusion, we demonstrated that cathepsin G and neutrophil elastase, normally synthesized in precursors cells of the bone marrow, are subjected to processing before and after sorting into lysosomes. The mechanism(s) by which these neutral proteases are targeted to lysosomes remains unknown. The modifications demonstrated during targeting may play a role in activation of the proteases. Finally, unprocessed cathepsin G and neutrophil elastase were secreted even when oligosaccharide processing was abnormal.

REFERENCES

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