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Biosynthesis of Lysosomal Endopeptidases

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Despite the clear differences between the amino acid sequence and enzymatic specificity of aspartic and cysteine endopeptidases, the biosynthetic processing of lysosomal members of these two families is very similar. With in vitro translation and pulse-chase analysis in tissue culture cells, the biosynthesis of cathepsin D, a aspartic protease, and cathepsins B, H and L, cysteine proteases, are compared. Both aspartic and cysteine endopeptidases undergo cotranslational cleavage of an amino-terminal signal peptide that mediates transport across the endoplasmic reticulum (ER) membrane. Addition of high-mannose carbohydrate also occurs cotranslationally in the lumen of the ER. Proteases of both enzyme classes are initially synthesized as inactive proenzymes possessing amino-terminal activation peptides. Removal of the propeptide generates an active single-chain enzyme. Whether the single-chain enzyme undergoes asymmetric cleavage into a light and a heavy chain appears to be cell type specific. Finally, late during their biosynthesis both classes of enzymes undergo amino acid trimming, losing a few amino acid residues at the cleavage site between the light and heavy chains and/or at their carboxyltermini. During biosynthesis these enzymes are also secreted to some extent. In most cells the secreted enzyme is the proenzyme bearing some complex carbohydrate. Under certain physiological conditions the inactive secreted enzymes may become activated as a result of a conformational change that may or may not result in autolysis. Analysis of the biochemical nature of the various processing steps helps define the cellular pathway followed by newly synthesized proteases targeted to the lysosome.

Key words: lysosomal enzymes; cathepsin D, B, L, H; protease; protease biosynthesis; proteolytic processing; carbohydrate; posttranslational processing

Mammalian lysosomal endopeptidases are intracellular catabolic enzymes of broad specificity. The majority are small monomeric glycoproteins ranging in molecular weight from 20 to 45 kDa. Cysteine proteases comprise the largest family by far. Of this group, cathepsin B, H, and L have been well characterized, while a variety of others have been described but have not been sequenced [reviews, 1–3]. Cathepsin L has previously also been known as mouse cysteine protease of activated macrophages

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[4] and the major excreted protein of transformed mouse fibroblasts [5]. It is now clear that these are three names for biosynthetic forms of the same protein. In contrast to cysteine proteinases, only a single lysosomal aspartic protease, cathepsin D, has been extensively studied [review, 6]. A second aspartic protease, cathepsin E, has been reported [7] to be localized in lysosomes in bone marrow polymorphonuclear leukocytes and macrophages but has not been sequenced. No lysosomal metallo-endopeptidases are known, but a number of lysosomal cysteine, serine and metallo-exopeptidases have been described [8]. Characterization of lysosomal proteases has been slow relative to secreted proteases because the lysosomal enzymes comprise only 0.01 to 0.001% of the total cellular protein. Only four mammalian lysosomal proteases have been cloned (cathepsin B [9–11], cathepsin D [12], cathepsin L [4,13–16], and cathepsin H [17]); no crystal structures have been reported. Curiously, no lysosomal storage disease has been ascribed to protease loss, in contrast to the lysosomal glycosidases. Presumably either loss of a particular lysosomal protease is fatal, or more likely, numerous enzymes of overlapping specificities have evolved precisely because protease activity is so essential.

This review compares the biosynthesis of three mammalian cysteine proteases, cathepsins B, H, and L, to that of the lysosomal aspartic protease cathepsin D. Like all lysosomal enzymes, these proteases undergo extensive proteolytic and glycosidic modification before reaching the lysosome (Fig. 1). Our improved understanding of the biosynthetic forms of these enzymes should aid future studies of the distribution and role of these proteases in various cellular functions.

SIGNAL PEPTIDE CLEAVAGE

All known luminal lysosomal proteases are synthesized with transient amino-terminal signal peptides that mediate their transport across the endoplasmic reticulum (ER). These early events in enzyme biosynthesis have been extensively characterized for cathepsin D. In vitro translation studies [18,19] coupled with radiosequence analysis [20] demonstrated that the initial events in the biosynthesis of lysosomal enzymes are indistinguishable from those for secretory proteins. Cathepsin D is synthesized with a 20-amino-acid amino-terminal signal peptide that is similar in length and charge distribution to those on secretory proteins. This form can only be detected in vitro and cannot be immunoprecipitated from cells. Transport of the preprotein across microsomal membranes is receptor mediated, occurring through interaction of the sig-

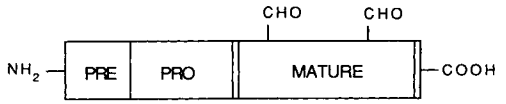

Fig. 1. Lysosomal protease biosynthesis. The biosynthetic processing of lysosomal aspartic and cysteine proteins is illustrated diagrammatically. Vertical lines indicate proteolytic processing steps. The number of carbohydrate chains added and the exact site of cleavage vary with the enzyme and cell type. Cleavage of an amino-terminal signal peptide and addition of carbohydrate occur cotranslationally, whereas the remaining processing steps occur posttranslationally.

nal recognition particle with the signal peptide and the ER membrane [21]. The signal is cleaved cotranslationally by luminal signal peptidase and two chains of high-mannose carbohydrate are added [20].

Similarly, a 17-amino-acid signal peptide has been identified on mouse cathepsin L through alignment of a cDNA sequence with protein sequences obtained for pro cathepsin L [16,76]. As seen for cathepsin D [12,20], cleavage of the signal occurs after an Ala-X-Ala sequence, a common cleavage site for signal peptides [22]. Cathepsin B has been predicted to have a 17-amino-acid [10] and cathepsin H a 21-amino-acid [17] signal sequence, both of which are cleaved after an Ala-X-Ala sequence. Consistent with these predictions, in vitro translation studies have shown that cotranslational transport of cathepsin H across the ER membrane is coupled with carbohydrate addition and loss of a 2 kDa peptide [23], presumably a signal peptide.

PROPEPTIDE CLEAVAGE

All of the well-characterized lysosomal proteases are synthesized with amino-terminal propeptides. The existence of pro forms of these enzymes was first observed for cathepsin D. The form detected on in vitro translation [18,19] and in vivo pulse-chase experiments [19,20,24,25] was found to be considerably larger than the well-characterized active form of the protease isolated preparatively from tissue [26]. Partial amino terminal–sequence analysis of radiolabeled early biosynthetic forms of the enzyme resolved on polyacrylamide gels revealed the presence of a 44-amino-acid propeptide [20]. Subsequently, alignment of cDNA sequences with protein sequences obtained for enzymes isolated preparatively from tissue has demonstrated the presence of a 62-amino-acid amino-terminal propeptide on human cathepsin B [10], a 96-amino-acid propeptide on cathepsin L [4,14,16], and a 92-amino-acid pro region on cathepsin H [17]. The presence of a propeptide on lysosomal proteases is not unexpected, given that secreted proteases such as trypsin and pepsin are also made as pro enzymes.

No significant sequence similarity is seen between the propeptides of cathepsin D and the cysteine proteases. The propeptides of cathepsin B from various species are 68–90% identical [10], which is consistent with the 64% identity of the propeptides of secreted pepsinogens from different species [12]. Yet the cathepsin D propeptide is only 43% identical with these propeptides of the pepsinogens [12], and the propeptide of cathepsin H shows only 21–34% identity, compared with the propeptides of other cysteine proteases [17].

Unlike both peptide hormone and neuropeptide precursors [27], propeptide cleavage does not occur at pairs of basic amino acids. Comparison of propeptide cleavage sites of lysosomal proteases (Fig. 2) reveals no obvious conserved characteristic that might be recognized by the processing enzyme. This suggests that the initial cleavage may occur closer to the amino terminus and be followed by rapid exopeptidase cleavage of residues from the amino terminus of the mature form. Such enzymes usually stop at proline, the second residue of both mature cysteine proteases and mature cathepsin D. On the basis of inhibitor studies, Hara and coworkers [29] have suggested that a metallo-endopeptidase is responsible for propeptide cleavage from cathepsins B, H, and L, whereas Nishimura and collaborators [30–32] propose that

Human Cathepsin D	VTE ↓ GPI
Mouse Cathepsin B	DID ↓ LPE
Rat Cathepsin B	DIN ↓ LPE
Human Cathepsin B	DLK ↓ LPA
Rat Cathepsin H	TGP ↓ YPS
Mouse Cathepsin L	MLK ↓ IPK
Rat Cathepsin L	MLQ ↓ IPK
Human Cathepsin L	FYE ↓ APR

Fig. 2. Propeptide cleavage sites. The known sites of lysosomal protease propeptide cleavage for human cathepsin D [12]; rat, mouse, and human cathepsin B [10]; rat cathepsin H [17]; mouse cathepsin L [4,16,76]; rat cathepsin L [14]; and human cathepsin L [28] are compared. The absence of obvious conserved features suggests that the initial cleavage may occur closer to the amino terminus.

cathepsin D is the processing protease. The subcellular site of propeptide cleavage is uncertain, but cleavage probably initiates in a vesicle before mature lysosomes [25,33].

It seems likely that the propeptides on lysosomal proteases, in analogy to the propeptides of secreted proteases, are involved in regulation of enzyme stability and activity. It would clearly be beneficial to a cell to synthesize and transport these enzymes as inactive proteases. Yet there is increasing evidence to suggest that under certain conditions proteases may undergo activation without cleavage of the propeptide (see below, Secretion of Lysosomal Proteases).

LIGHT-HEAVY CHAIN CLEAVAGE

In some but not all cell types the mature single-chain protease is seen in pulse-chase studies to be cleaved asymmetrically into a two-chain form (Fig. 3). The rate of this cleavage step is variable. In mouse macrophages, conversion of cathepsin L to the two-chain form is rapid, whereas cleavage of cathepsins B and H occurs more slowly [35]. The ratio of single-chain enzyme to two-chain form varies with the species and with the purification technique. Cathepsin L exists in the two-chain form in mouse peritoneal macrophages but not in the macrophage-like mouse WEHI-3 cell line [76]. Chicken liver cathepsin L is primarily a single-chain enzyme [36]. Tang and coworkers [26] found that bovine cathepsin D contains about 68% of the single-chain species, whereas the porcine kidney enzyme contains only 5%. In rat tissues, in contrast, cathepsin D exists entirely in a single-chain form [37] (Erickson, unpublished).

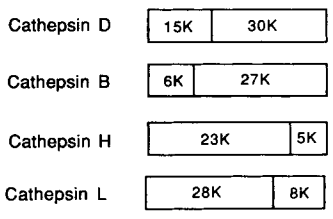

Fig. 3. Generation of light and heavy cathepsin chains. Asymmetric cleavage of active single-chain cathepsins produces two-chain forms. Chain molecular weights are illustrated for porcine cathepsin D [20], human cathepsin B [34], rat cathepsin H [35], and mouse cathepsin L [4].

Human Cathepsin D	VSVPCQ↓SASSASA↓LGG
Human Cathepsin B	ICIHTN↓AH↓VSVEVSAE
Rat Cathepsin B	ICIHTN↓GR↓VNVEVSAE
Rat Cathepsin H	YGEQN↓GLLYWI
Human Cathepsin L	EST↓ESD↓NNKYWL
Mouse Cathepsin L	EGT?DSN↓KNKYWL
Rat Cathepsin L	EGT?DS↓NKDKYWL

Fig. 4. Sites of cleavage of single-chain cathepsins. This cleavage does not occur for all lysosomal proteases in all cell types. The sites of asymmetric cleavage of active human cathepsin D [12,26], human cathepsin B [10,39], rat cathepsin B [10,38], rat cathepsin H [17,38], human cathepsin L [28,40], mouse cathepsin L [4,16,76], and rat cathepsin L [14] are compared. The human cathepsin L cleavage sites are based on protein and cDNA sequencing; the first mouse site is predicted only by analogy with the human, whereas the first rat cathepsin L cleavage is predicted on the basis of amino-acid analysis of the rat light and heavy chains.

Alignment of the known cleavage sites (Fig. 4) indicates that cleavage usually occurs after an amino acid (asparagine, glutamine) having an amide side chain for both aspartic and cysteine proteases. More examples will be necessary, however, to establish that the amide side chain plays a critical role in site recognition by the cleavage enzyme. Leupeptin has been reported to block this proteolytic processing step for cathepsin D [6], human cathepsin B [34], and rat cathepsins B, H, and L [29], suggesting that a cysteine protease may be involved. In mouse cathepsin L (Erickson and Portnoy, unpublished), but not in porcine cathepsin D [26], the two chains are held together by disulfide bonds.

The functional significance of this cleavage is unclear. It is difficult to distinguish if the single chain is merely cleaved as a result of the copackaging with other lysosomal proteases, perhaps as a late breakdown step, or if the cleavage serves a physiologically significant function. The single-chain and two-chain forms appear to be equally active for cathepsin D [26]. Blocking this cleavage with leupeptin [34], however, leads to an increase in the specific activity of cathepsin B in human fibroblasts, suggesting that the single-chain enzyme may be the more active form.

AMINO ACID TRIMMING

Very late during their biosynthetic processing, most lysosomal proteases undergo proteolytic trimming. This process is detectable as a molecular-weight decrease of 1–2 kDa approximately 24 h after synthesis in tissue culture cells [41]. This late shift in molecular mass can be due to one or two kinds of proteolytic processing (Figs. 4,5).

First, if the single-chain enzyme is cleaved to a two-chain form, several amino acids may be lost, usually from the amino terminus of the chain derived from the carboxyl terminus of the single chain. This is detectable by comparing the cDNA sequence to the protein sequence of the mature enzyme isolated preparatively from tissue. By this method, cathepsin D was found to lose seven amino acids between the light and heavy chains [12,26] and cathepsin B was found to lose two amino acids from this site [10,38]. Similarly, human cathepsin L [39,40] and probably mouse

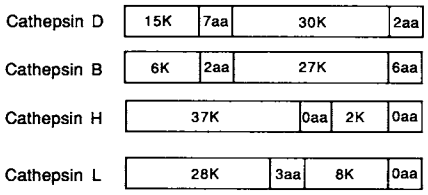

Fig. 5. Trimming of amino acids from cathepsin chains. The number of amino acids trimmed off cathepsin D [12,26,41], cathepsin B [10,11,38], cathepsin H [17,38], and cathepsin L [28,40] are illustrated. These were determined by comparison of a cDNA sequence with a protein sequence obtained on a late biosynthetic form of preparatively isolated enzyme.

cathepsin L [4,16,40] lose three amino acids between the heavy and light chains, while the rat enzyme evidently loses only two [14]. In contrast, rat cathepsin H does not lose any amino acids on cleavage of the single-chain form into heavy and light chains [17]. It is not known whether this amino-acid trimming occurs immediately on chain cleavage or is a later processing event.

Second, some lysosomal proteases have been found to undergo limited trimming at their carboxyl terminus very late during biosynthesis (Fig. 5). This pattern was first established by demonstrating a difference in the rate of release of a particular radiolabeled amino acid from the carboxyl terminus of early and late biosynthetic forms of cathepsin D [41]. Comparison of the protein sequence deduced from the cDNA sequence to the protein sequence obtained for preparatively isolated enzyme demonstrated that human cathepsin D loses two amino acids [12,26] and that rat and human cathepsin B each lose six amino acids from their carboxyl termini [10,11,38]. Comparison of the protein sequence deduced from the cDNA sequence of human cathepsin L [28] to the protein sequence of human cathepsin L [40] suggests that human cathepsin L does not lose carboxyl-terminal amino acids. Likewise, a similar comparison of sequences did not reveal any carboxyl-terminal trimming of rat cathepsin H [17].

The physiological significance of this late step is unknown. It may only occur if the vesicle pH drops significantly, thereby activating a particular protease. This may occur after a lysosome has fused with a phagosome. The processing step could play a role in the turnover of lysosomal enzymes, which eventually must themselves be degraded.

SECRETION OF LYSOSOMAL PROTEASES

All lysosomal enzymes are secreted to some extent, but the amount of enzyme released varies greatly with the cell type and culture conditions. Raising the pH of lysosomes by treating cells with ammonium chloride or chloroquine induces secretion of most of the newly synthesized proenzymes [24,42]. It is not clear how the amount of enzyme released from tissue culture cells correlates with the amount of enzyme secreted from cells in vivo. Reports, for example, of cathepsin D in serum and urine [43] and of cathepsin B–like activity in extracellular fluids [44–46] indicate that secretion is not merely a result of cell culture.

The secreted form is the proenzyme that has lost its amino-terminal signal peptide but that has not undergone further proteolytic processing. Endoglycosidase treatment of enzymes isolated from cell culture medium reveals that the majority of the secreted enzymes, like most secretory proteins, possess complex carbohydrate. Unlike secretory proteins, the secreted lysosomal enzymes all bear the mannose-6-phosphate lysosomal recognition marker and therefore are able to be taken back into cells through interaction with cell surface mannose-6-phosphate receptors [reviews, 47,48]. It is not clear why a certain percentage of newly synthesized enzyme escapes interaction with intracellular mannose-6-phosphate receptors and subsequent transport to lysosomes.

At the neutral pH of the extracellular environment, most secreted lysosomal proenzymes are inactive and/or unstable. In contrast, for example, the early biosynthetic form of the lysosomal glycosidase beta-hexosaminidase is catalytically active in the cell medium [24]. Yet measurements with microelectrodes have shown that the microenvironment at the surface of activated macrophages is acidic, with pH values of less than 5 [49]. If local areas of low pH do exist around cells and tissues, then the proteases may become activated. Exposure to low pH may cause a conformational change that induces autocatalytic activation of the enzyme by propeptide cleavage, a well-documented process for the secreted protease pepsin [50]. For both cathepsin D [51] and cathepsin L [52], incubation at low pH results in a decrease in molecular mass, as judged by SDS gel electrophoresis and a concomitant increase in enzymatic activity. Similarly, a precursor form of cathepsin D secreted by human breast cancer cells has been found to be enzymatically active after autoactivation by cleavage of a 1-kDa peptide [53].

Alternatively, a conformational change unaccompanied by proteolysis may also activate lysosomal proenzymes. Exposure to pH 3.5 has been found to induce procathepsin D to bind to pepstatinyl-agarose, whereas at pH 5.3 only mature forms of cathepsin D bind [6]. Similarly, cathepsin L, found to be secreted in increased quantities by transformed mouse 3T3 cells, can be activated without proteolysis. This secreted form, originally named MEP for major excreted protein [5], is procathepsin L, indistinguishable from the intracellular proenzyme. Secreted procathepsin L is stable over the pH range of 4.5–8 and active when local pH values fall below 6.0 [54]. Human malignant breast tumors in organ culture release a high-molecular-weight form of cathepsin B that is thought to be enzymatically active [55]. Murine B16 melanoma variants of different metastatic potential, however, secrete cathepsin B that is 84–97% latent [56].

Clearly, the extracellular activation of secreted lysosomal proenzymes, because of either autolysis or a conformational change causing enzyme activation, can have physiological significance. Sloane and Honn [57] have demonstrated a correlation between extracellular levels of cathepsin B–like activity and tumor metastasis. Denhardt and coworkers have found a correlation between cathepsin L secretion and metastatic potential in mouse H-*ras*-transfected cell lines [58]. Briozzo and coworkers have recently demonstrated that cathepsin D secreted by human breast cancer cell lines can degrade extracellular matrix after autoactivation at pH 4–5 [59]. Also, cathepsin L has been shown to have elastinolytic activity at pH 5.5 [60] and to be capable of interfering with antigen processing by destroying antigenic determinants after being internalized into an acidic compartment [61].

Thioglycollate-elicited mouse peritoneal macrophages differ from other cell types, including resident peritoneal macrophages, in that they secrete proteolytically processed active forms of cathepsin L [76] and of the lysosomal glycosidases beta-glucuronidase and beta-galactosidase [62]. Similarly, zymosan-treated human macrophages release processed forms of cathepsin D [63]. It is unclear if in these cells the lysosomes fuse with the plasma membrane and release their contents to the extracellular environment directly or if release occurs only during the process of phagocytosis.

CARBOHYDRATE CHAINS

During translocation across the ER membrane, newly synthesized lysosomal enzymes acquire high-mannose carbohydrate. The number of chains added varies with the particular protein. Attachment can, but does not always, occur at the asparagine of Asn-X-(Ser/Thr) sites [review, 64]. Cathepsin D bears two carbohydrate chains, one on the heavy chain and one on the light chain [65], and an additional potential site is present on the propeptide [12]. Cathepsin B has one carbohydrate chain on its heavy chain [66,67] and one potential site on its propeptide [10]. Also, cathepsin H has one site on the mature enzyme [66,67] and two potential sites on the propeptide [17]. It is not known if the sites on the propeptides are actually all glycosylated, but endoglycosidase treatment of cathepsin B and H suggests that they are [68]. In contrast, cathepsin L has no glycosylation site on its propeptide and two acceptor sites on the active protein [4,16], only one of which is glycosylated [76].

In general, intracellular lysosomal proteases have primarily trimmed high-mannose carbohydrate, whereas the secreted forms have complex carbohydrate. These two types of carbohydrate can be distinguished by their different susceptibility to endoglycosidase cleavage. The oligosaccharides of cathepsin B are smaller than those on other lysosomal enzymes [66,67], presumably because of extensive trimming of the original high-mannose chain. The carbohydrate on cathepsin D has been found to contain sulfate transiently, but the functional significance of this posttranslational modification is unclear [69]. In the Golgi, phosphate is added to certain mannose residues to form the mannose-6-phosphate recognition marker, which allows lysosomal enzymes to bind to the receptors that transport the enzymes to lysosomes [reviews, 47,48].

FUNCTION AND REGULATION OF EXPRESSION

Accumulating recent evidence indicates that expression of lysosomal proteases may be regulated. Tissue levels of cathepsin B are variable, as measured by histochemical staining [70] or Northern blots of mRNA [71], suggesting that this protease may participate in specialized cellular functions. Distribution of cathepsin L has also been found by Northern blot analysis to vary with the cell type [4]. Yet by the same technique, cathepsin D levels were found to be relatively uniform between tissues [71]. The extensive trimming of the cathepsin B carbohydrate and the lack of amino-acid trimming after cleavage of the single-chain form of cathepsin H distinguish these two enzymes from the other lysosomal endopeptidases and raise questions concerning their intracellular localization and function. Recent work by P. Stahl and coworkers [72,73] indicates that cathepsin D exists in rabbit aveolar macrophage endosomes in an active

membrane-bound state before conversion to the soluble form found in lysosomes. This intriguing finding raises questions about the roles of this enzyme in endosomes and mature lysosomes. Finally, cathepsins D, B, and L have all been found to be secreted in increased amounts by certain tumor cells [5,53,55,74,75], suggesting that the lysosomal proteases may play an active extracellular role in cancer metastasis and tissue remodeling. These findings together clearly suggest that lysosomal endopeptidases may play physiological roles discrete from or in addition to their role in protein turnover in the lysosome.

FUTURE DIRECTIONS

To reach the correct cellular destination, a lysosomal protein must undergo certain processing steps in a defined order. To undergo the correct processing, an enzyme must possess structural features that are recognized by the processing enzymes. Essentially nothing is known about these critical sites on the surfaces of lysosomal proteases or about the enzymes that recognize them. Future efforts will be directed toward understanding what kinds of enzymes are responsible for the processing steps, which features they recognize on lysosomal enzymes, and where in the cell they are located. These studies promise to reveal the physiological role of the various processing events.

This understanding of lysosomal enzyme biosynthesis is the basis for studies aimed at determining the role lysosomal proteases play in abnormal physiological states. It is becoming clear that increased synthesis of lysosomal proteases can be induced and that the enzymes can be secreted to the extracellular environment, where they may play roles in tumor metastis or muscle readsorption, for example. Yet, at present, we do not know how the synthesis of a particular protease is regulated, why the enzyme is secreted when it carries a mannose 6-phosphate recognition marker, or how it becomes activated in the extracellular environment. Efforts to control the release and activation of these potentially destructive proteases must be based on a clear understanding of their basic biosynthesis.

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