# Chapter 4
# Cathepsins: Getting in Shape for Lysosomal Proteolysis

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## 4.1 Introduction

Besides their pivotal functions in general cellular protein turnover, cathepsins play important roles in a diverse range of other physiological processes which include tissue remodelling during embryogenesis and development, programmed cell death, autophagy, prohormone and neuropeptide processing, antigen presentation, wound healing and bone resorption. Furthermore, substantial experimental evidence has been accumulated that cathepsins are of pathological relevance in disease states such as cancer, arthritis, osteopetrosis, pancreatitis, cholestatic liver disease, and epilepsy (Mohamed and Sloane 2006; Vasiljeva et al. 2007; Turk and Turk 2009; Reiser et al. 2010). To prevent tissue damage due to unwanted proteolysis, the activities of cathepsins have to be strictly controlled *in situ*. The main regulatory pathways rely on restricting the subcellular localization of these proteases to lysosomes, the presence of specific cathepsin inhibitors in other cellular compartments, and their initial synthesis as latent proenzymes (Cygler and Mort 1997; Mort and Buttle 1997;

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Turk et al. 2001b). Interference with any of these control mechanisms can lead to pathological consequences. It is therefore crucial to understand the molecular basis of cathepsin biosynthesis and intracellular transport as well as the mechanisms leading to activation of their precursors in a cellular context.

## 4.2 Mannose 6-Phosphate Receptors: Key Cellular Interaction Partners of Lysosomal Cathepsins

The extracellular accumulation of various cathepsins in cancer, arthritis and other human disorders indicates disease-associated changes in the biosynthesis and intracellular transport of these proteinases. However, extensive studies on the biosynthesis of individual cathepsins have been performed only for three of them: cathepsin B, cathepsin D, and cathepsin L. These proteinases are synthesized and targeted to their final intracellular destination in a similar manner as most other soluble lysosomal proteins, involving the following general steps: synthesis as latent preproenzymes by ribosomes associated with the rough endoplasmic reticulum (ER), translocation into the lumen of this compartment, delivery to and passage through the different stacks of the Golgi apparatus, and finally receptor-mediated transport from the *trans*-Golgi network (TGN) to lysosomal compartments followed by receptor-ligand dissociation and proteolytic maturation of the proteinase precursors *in situ* (Erickson 1989; Hasilik 1992; Mach 2002).

Like most other soluble lysosomal enzymes, cathepsins are glycoproteins which are modified in their *N*-glycosidically linked oligosaccharide chains with mannose 6-phosphate (M6P) residues. This unique post-translational modification is critical for the correct intracellular targeting of the proteinases. Two enzymes are responsible for the creation of the M6P recognition marker: UDP-*N*-acetylglucosamine-1-phosphotransferase (phosphotransferase) and *N*-acetylglucosamine-1-phosphodiester α-*N*-acetyl-glucosaminidase (uncovering enzyme). Phosphotransferase, a *cis*-Golgi enzyme, attaches GlcNAc-1-phosphate residues from UDP-GlcNAc to the C-6 hydroxyl group of selected mannoses in the high mannose-type oligosaccharides of newly synthesized lysosomal hydrolases (Reitman and Kornfeld 1981; Tiede et al. 2005). This generates a phosphodiester, Man-P-GlcNAc. The sugar moiety masking the phosphate group is then removed by the uncovering enzyme localized in the TGN (Waheed et al. 1981; Rohrer and Kornfeld 2001). This exposes the phosphomonoester residue that is then recognized by specific M6P receptors residing in the same compartment, which then deliver the newly synthesized enzymes to lysosomes (Braulke and Bonifacino 2009; Saftig and Klumperman 2009).

Phosphotransferase is unique in its requirement for specific oligosaccharide acceptor sites only present in lysosomal proteins. The fundamental importance of this enzyme for lysosome biogenesis is documented by the fact that its deficiency leads to the inherited disorder mucolipidosis II, also referred to as I-cell disease

(Tiede et al. 2005). It is still unclear how phosphotransferase distinguishes between lysosomal and secretory proteins. However, it has been proposed that its interaction with cathepsins B, D and L involves the recognition of a structural motif based on distinct spatial positioning of certain lysine residues (Cuozzo et al. 1998; Lukong et al. 1999). At least for fibroblast cathepsin B, modification with M6P residues seems mandatory for delivery of the enzyme to lysosomes, since cells from I-cell disease patients almost quantitatively fail to retain their newly-synthesized procathepsin B (Hanewinkel et al. 1987). The latter is also observed in the case of murine fibroblasts lacking both M6P receptors (Probst et al. 2006).

Two distinct M6P-binding proteins occur in mammalian cells, the 300-kDa mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) and the 46-kDa cation-dependent mannose 6-phosphate receptor (MPR46). The comparison of their cDNA sequences has revealed that the two receptors are related proteins. While M6P/IGF2R has an extracytoplasmic domain consisting of 15 repeating segments, the entire extracytoplasmic domain of MPR46 is similar to each of the repeating units of the former protein. This suggests that the two receptors may be derived from a common ancestor (Ghosh et al. 2003; Dahms et al. 2008). Fibroblasts devoid of both receptors have a phenotype similar to cells from patients with mucolipidosis II, quantitatively secreting most of their newly synthesized lysosomal enzymes. In cells lacking only one of the two proteins, the remaining receptor cannot fully compensate for the absence of the other (Ludwig et al. 1994; Pohlmann et al. 1995). Interestingly, hypersecretion of lysosomal enzymes by cells lacking M6P/IGF2R can be only partially rescued by overexpression of MPR46 (Watanabe et al. 1990).

Besides sorting newly synthesized lysosomal enzymes from the Golgi apparatus to lysosomes, M6P receptors also function in the endocytosis of extracellular lysosomal enzymes. It is remarkable that M6P/IGF2R, but not MPR46, is capable of mediating this process. Thus, mammalian cells have two means to deliver M6P-tagged proteins to lysosomes: (a) the biosynthetic route via vesicles derived from the Golgi apparatus, and (b) recapture of mistargeted and hence secreted lysosomal enzymes via the endocytic route, with the latter process being strictly dependent on M6P/IGF2R. It has been proposed that the secretion-recapture pathway contributes significantly to the efficiency of lysosomal enzyme sorting in mammalian cells (Kasper et al. 1996). Taken together, this indicates that two distinct M6P receptors are engaged in cathepsin trafficking to lysosomes, with M6P/IGF2R being the major protein responsible for this important cellular process.

The displacement of M6P-modified cathepsins from their sorting receptors occurs upon reaching endosomes, followed by rapid M6P hydrolysis due to the action of phosphatases. Receptor-ligand dissociation is thought to be due to the reduced affinities of M6P receptors for their ligands at the low pH of the endosomal lumen. In cells expressing both M6P receptors, agents that perturb endosomal acidification have been shown to interfere with cathepsin trafficking to lysosomes (Braulke et al. 1987). However, such lysosomotropic amines do not noticeably affect the residual intracellular retention of cathepsins B, D and L in fibroblasts and epithelial cells lacking M6P/IGF2R. Intriguingly, intracellular transport of

cathepsin B in M6P/IGF2R-deficient cells is still entirely dependent on the presence of the M6P recognition marker, indicating that it is mediated by MPR46. These findings strongly suggest that at least dissociation of MPR46 and its ligands can also occur in the absence of endosomal acidification (Probst et al. 2006).

Although the M6P receptor system is the main pathway for lysosomal enzyme sorting, it has become evident that in specialised cell types intracellular transport of soluble acid hydrolases to lysosomes can also occur in an M6P-independent manner. The strongest support for the existence of M6P-independent lysosomal trafficking pathways comes from studies involving cells of lymphocytic origin. In lymphoblasts from I-cell disease patients, transport of cathepsin D to lysosomal compartments is not dependent on *N*-glycosylation of the protein (Glickman and Kornfeld 1993). Cathepsin B was also localized in lysosomal compartments of I-cell disease lymphoblastoid cell lines (Griffiths and Isaaz 1993). Moreover, murine thymocytes deficient in both M6P receptors retain their newly-synthesized procathepsin D as efficiently as their normal counterparts (Dittmer et al. 1999). Recently, evidence has been provided that sortilin could be involved in M6P-independent targeting of cathepsin D to lysosomes (Canuel et al. 2008). Alternatively, M6P-independent transfer of cathepsins to lysosomes could rely on a secretion-recapture pathway, possibly engaging cell-surface lectins such as the asialoglycoprotein receptor or the mannose receptor. M6P-independent uptake of secreted cathepsins might also occur by means of direct cell-to-cell contact, involving pinocytotic microinvaginations and non-coated vesicles (Dittmer et al. 1999).

Cathepsins are delivered to endosomes as latent proenzymes. It is generally believed that proteolytic maturation of the precursors is initiated once receptor-mediated delivery to endosomal compartments has been achieved. Proteinase activation is then triggered by the local acidic environment. It has been demonstrated that purified procathepsins B, D and L can undergo autocatalytic activation in acidic conditions (Hasilik et al. 1982; Mach et al. 1994a; Ménard et al. 1998). Activation is quickly followed by cleavage of the latent proenzymes into the single-chain forms of the respective proteinases (see chapter 4.5 for details). Finally, terminal processing of the single-chain enzymes into the corresponding double-chain forms occurs as a late biosynthetic event in the lysosomes.

## 4.3 Cysteine Cathepsins: Endopeptidases and Exopeptidases

Protein degradation in lysosomes and related compartments is thought to involve two main phases. The initial digestion of the substrates is achieved by the action of cathepsins with endopeptidase activity. The fragments thus generated are then converted by exopeptidases into small peptides and free amino acids. Three endopeptidases appear to be present in all mammalian lysosomes: the aspartic proteinase cathepsin D, and the cysteine proteinases cathepsin B and cathepsin

L. Cathepsin B also displays dipeptidylcarboxypeptidase activity. In contrast, cathepsin D and cathepsin L are classical proteinases lacking any exopeptidolytic potential (Brix et al. 2008; Masson et al. 2010).

Cathepsin B and cathepsin L are members of the so-called papain superfamily of lysosomal cysteine proteinases, which includes another nine human enzymes: cathepsins C, F, H, K, O, S, V, W and X. Two ubiquitously expressed genuine exopeptidases without endopeptidolytic activity belong to this family, the dipeptidylaminopeptidase cathepsin C and the carboxypeptidase cathepsin X. Cathepsin H exhibits both aminopeptidase and endopeptidase activity. Some specialised cell types express other family members with close homology to cathepsin L. The best studied of these tissue-specific lysosomal cysteine proteinases are cathepsins K and S (Turk et al. 2000; Brix et al. 2008).

Of the 11 human cysteine cathepsins, 7 appear to be obligate endopeptidases. Three of these—cathepsins K, L and S—have been studied extensively whereas cathepsins F, O, V and W have received less attention. The mature forms of these proteases consist of a two-lobed papain-like structure, the junction between which forms the substrate-binding cleft. This channel is usually capable of housing three amino acid residues on the unprimed side of the peptide linkage targeted for cleavage and at least two residues on the primed side (Turk et al. 1998; Stern et al. 2004). The major selectivity of the cysteine cathepsins lies in the P2 position. As endopeptidases, cathepsins K, L and S are very efficient protein dismantling agents.

Building on the basic papain model, additional structural elements have evolved to restrict the active site cleft so that the resulting enzyme becomes one of four exopeptidase types. Cathepsins B and C remove dipeptides from the C- or N-terminus, while cathepsins X and H are monocarboxy- and aminopeptidases, respectively. The carboxypeptidase activities are mediated by the inclusion of an extra loop in the basic structure of the mature protease. Termed the occluding loop in the case of cathepsin B (Musil et al. 1991) and the mini loop in the case of cathepsin X (Nägler et al. 1999b), these elements block off the primed side of the active site cleft and position a histidine side chain to accept the negative charge on the substrate C-terminus. The size of the loop allows for the positioning of two or one residues, respectively, at the primed side of the site of cleavage. In cathepsin B the occluding loop is held in position by an ionic interaction between a second histidine residue in the loop and an aspartate residue located in the main body of the enzyme. Disruption of this interaction as occurs with increasing pH permits the loop to reorient (Nägler et al. 1997), and the enzyme then acts as an endopeptidase.

The molecular basis for aminopeptidase activity is more complex and depends on the positioning of remnants of the propeptides which restrict the unprimed side of the active-site cleft so that it can only accept one or two residues. In the case of cathepsin H an octapeptide mini-chain is disulfide bonded in place providing space for only a single residue before the cleavage site. The positive charge on the substrate N-terminus is matched by the carboxylate of the C-terminus of the mini-chain (Guncar et al. 1998). As would be expected, cathepsin H lacking the mini-chain is a functional endopeptidase (Vasiljeva et al. 2003). In cathepsin C a

119-residue β-barrel unit termed the 'exclusion domain' is employed for positioning of an aspartate side chain as the acceptor for the substrate N-terminus in the S2 pocket of the enzyme (Turk et al. 2001a). With this diversity of activities, the combined actions of the cysteine cathepsins can recycle a substrate protein to its constituent amino acids.

## 4.4 Structures of Cysteine Cathepsin Precursors

Proteolytic activity is inherently destructive and thus potentially hazardous to the cell. Proteases are therefore usually synthesized as inactive precursors which require processing to release functional enzymes at cellular locations where their action is required. Various strategies are employed by different protease families to realize this self-defense mechanism (Khan et al. 1998). In most cases the proenzyme contains an N-terminal propeptide extension which folds back onto the active site of the protease, thus blocking access to the substrate. The propeptides can be as small as a dipeptide as in the case of some serine proteases such as neutrophil elastase (Salvesen and Enghild 1990) and granzymes, or domains with over 90 residues as for various cysteine cathepsins.

In 1993, based on an analysis of the proenzyme sequences, the cysteine cathepsins were divided into two classes based on the presence of a highly conserved interspersed motif termed 'ERFNIN' (Karrer et al. 1993) which represents a series of residues predicted to lie along one surface of a large α-helix. Variants of this motif are present in the cathepsin L-like class members but absent in the cathepsin B-like enzymes. The three-dimensional structures of procathepsins K (LaLonde et al. 1999; Sivaraman et al. 1999), L (Coulombe et al. 1996) and S (Kaulmann et al. 2006) show a distinct α-helix-rich domain which is stabilized by a conserved tripartite tryptophan motif located at the interaction site between the two major α-helices (Kreusch et al. 2000), the second of which contains the ERFNIN motif. This double-helical domain is linked to the body of the catalytic unit by the insertion of an aromatic side chain into a hydrophobic pocket formed by a region termed the propeptide binding loop (Fig. 4.1). The structures of the proenzymes immediately explain their inability to cleave protein substrates since the propeptide extends through the active site blocking access to the catalytic machinery. Critically, the sense of the propeptide strand is reversed relative to that required for normal substrate binding, thus hampering processing of the propeptide (Cygler and Mort 1997). The inhibitory nature of the propeptide regions of these enzymes has been demonstrated by their production as independent modules. These can fold into helix-rich domains and were shown to be tight-binding inhibitors of their cognate enzymes (Schilling et al. 2009).

With 38 residues, the propeptide of cathepsin X is the shortest in the whole cysteine cathepsin family (Fig. 4.2), barely reaching beyond the active-site cleft (Sivaraman et al. 2000). However, its position is stabilized by a disulfide bond located between the prosegment and the active-site cysteine residue.

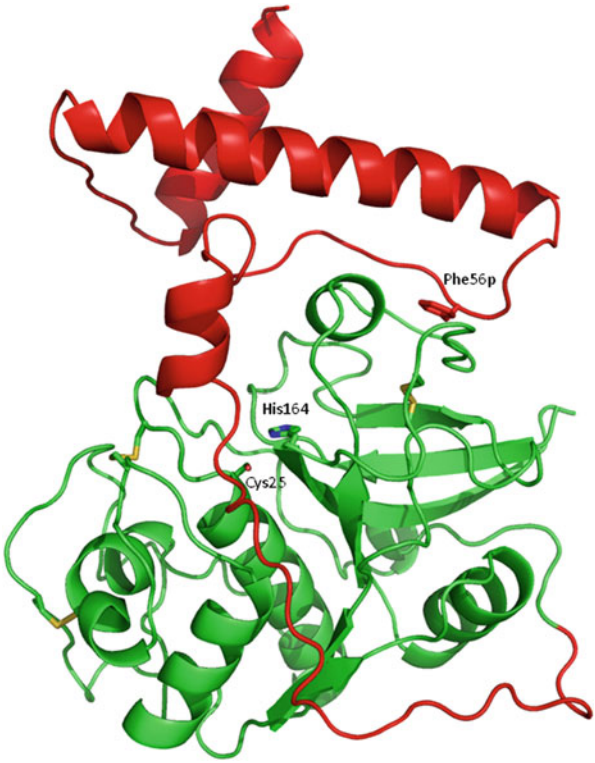


**Fig. 4.1** Three-dimensional structure of human procathepsin L. The propeptide is illustrated in *red* while the mature enzyme is in *green*. Disulfide bridges are displayed in *yellow*. The side chains of the catalytic cysteine and histidine residues are shown in *stick* representation, as is the side chain of the phenylalanine residue in the propeptide which anchors the propeptide to the mature enzyme through hydrophobic interactions (PDB 1CJL)

The propeptide of cathepsin B is intermediate in length between that of cathepsin X and those of the cathepsin L-like class. As in procathepsin L, an α-helical structural element is present which is bound to the protease through a hydrophobic interaction (Cygler et al. 1996; Turk et al. 1996). In mature cathepsin B, the occluding loop is positioned to accept the carboxylate of the P2′ substrate residue, as illustrated in the structure of a complex between the protease and the irreversible inhibitor CA-074 (Yamamoto et al. 2000) (Fig. 4.3b, c). In the proenzyme however, the position of the occluding loop is deflected by the propeptide (Fig. 4.3a). In fact, the occluding loop appears to be also adaptable in other situations such as the binding of the inhibitors chagasin (Redzynia et al. 2008) and cystatin A (Renko et al. 2010).

The large variation in the size of the cysteine cathepsin proregions suggests that the α-helical structural element may have additional functional roles. Some evidence for this is provided by the characterization of a receptor for procathepsin L (McIntyre and Erickson 1993) and binding of procathepsin B to annexin II (Mai et al. 2000). These interactions may play a role in the processing of the proenzyme forms or the targeting of the enzymes to lysosomes or other subcellular locations. Procathepsin F is a unique member of this family of enzymes since it contains a cystatin-like domain N-terminal to the cathepsin L-like propeptide region (Nägler

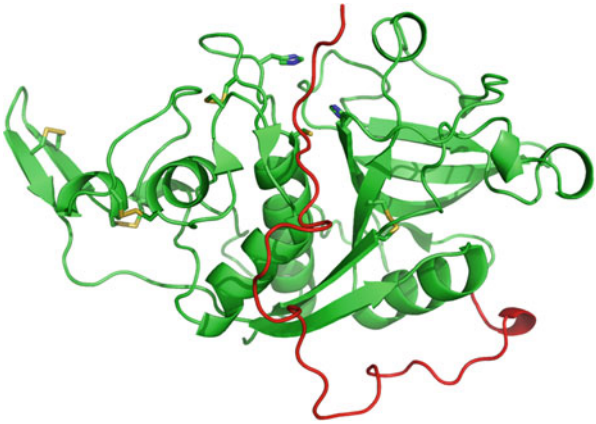

**Fig. 4.2** Three-dimensional structure of human procathepsin X. The propeptide is illustrated in *red* while the mature enzyme is in *green*. Disulfide bridges are displayed in *yellow*. The side chains of the catalytic cysteine and histidine residues are shown in *stick* representation, as is the side chain of the histidine residue of the mini-loop which is the acceptor for the C-terminal carboxylate of the substrate (PDB 1DEU)

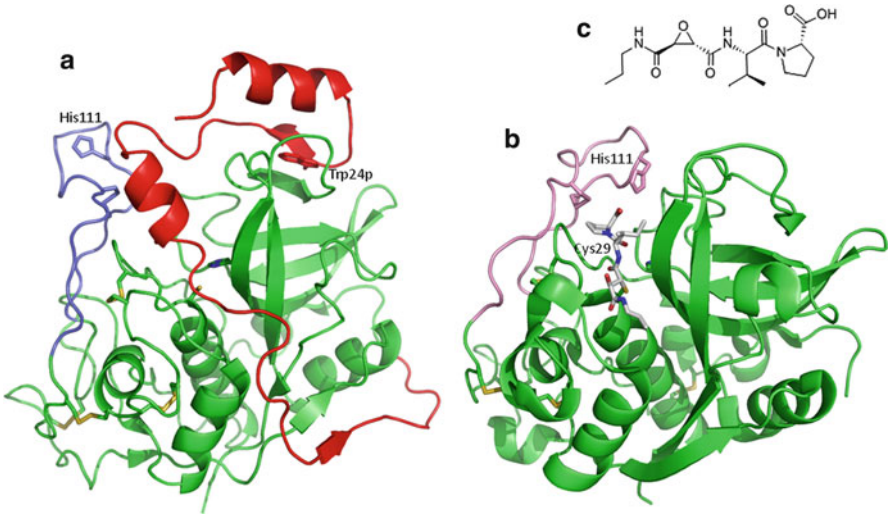


**Fig. 4.3** Three-dimensional structures of human procathepsin B and the mature enzyme. (**a**) Procathepsin B (PDB 3PBH) showing the propeptide in *red* and the mature enzyme in *green*. Disulfide bridges are displayed in *yellow*. The side chains of the catalytic cysteine and histidine residues are shown in *stick* representation, as is the side chain of the tryptophan residue in the proregion which anchors the propeptide to the mature enzyme through hydrophobic interactions. The occluding loop is illustrated in *blue*. Note that the loop is deflected by the presence of the propeptide. (**b**) A complex (PDB 1QDQ) between the mature protease and the irreversible inhibitor CA-074 (*trans*-epoxysuccinyl(propylamide)-Ile-Pro, chemical structure shown in **c**). The Ile-Pro portion of the inhibitor occupies the S1′ and S2′ subsites. The occluding loop is indicated in *pink*. The carboxylate of the Pro residue of the inhibitor interacts with the side chain of His111 located in the occluding loop

et al. 1999a; Wex et al. 1999). The functional significance of this domain is, however, still unclear.

The proregions of the exopeptidases follow the same overall strategy of using the propeptide to obstruct the active site cleft, but the N-terminal regions of the propeptides differ widely. No structural studies have been published so far for the proforms of cathepsins H and C. However, their sequences indicate that a similar crossed α-helix structure is present. This is also supported by a model of the procathepsin H structure (Horn et al. 2005).

## 4.5 Activation and Maturation of Cysteine Cathepsin Precursors

Activation of cysteine cathepsin precursors generally accompanies acidification of their environment as they are transported to the lysosome. Proteolytic cleavage of the proregion then occurs, and as was demonstrated in kinetic studies on the isolated proregions of cathepsins B (Fox et al. 1992), K (Billington et al. 2000) and L (Carmona et al. 1996), the binding affinity of the propeptide for its cognate enzyme decreases dramatically as the pH is lowered. Thus, once cleaved the propeptide dissociates from the catalytic unit and becomes a substrate for further proteolysis. Autoprocessing of several proenzymes has been observed in many cases and this can be facilitated by interactions with surfaces and with anionic polymers (Mason and Massey 1992; Caglic et al. 2007). Autoprocessing can occur through two possible mechanisms, intra-molecular or inter-molecular. Early studies using a procathepsin B in which the active-site cysteine residue had been mutated into serine demonstrated that processing can occur in *trans* with the propeptide being liberated by several lysosomal proteases including cathepsin B itself acting as an endopeptidase (Rowan et al. 1992). Claims of intra-molecular processing have been controversial (Rozman et al. 1999). Studies on the concentration dependence of autoprocessing of procathepsin B (Mach et al. 1994a), procathepsin L (Ménard et al. 1998) and procathepsin S (Quraishi and Storer 2001) clearly showed, by extrapolation of the observed rates to zero proenzyme concentration, that intra-molecular processing occurs, which probably requires the transient formation of short-lived processing intermediates (Quraishi and Storer 2001; Pungercar et al. 2009). While the propeptide passes through the active site in the reverse orientation to that required for normal substrate hydrolysis, the carbonyl residue of the peptide bond closest to the active-site cysteine is close enough to form a tetrahedral intermediate although this cannot be stabilized by the canonical oxyanion-hole mechanism. However, slow peptide-bond hydrolysis still occurs and this is followed by removal of the residual propeptide segment by inter-molecular processing. In contrast to cathepsin B and the members of the cathepsin L class, maturation of procathepsins X (Nägler et al. 1999b) and C (Dahl et al. 2001) requires the action of cathepsin L-like proteases.

## 4.6 Unconventional Cysteine Cathepsin Gene Products

For cathepsins B and L, isoforms have been described which lack parts of their respective propeptides. These variants are also devoid of a signal peptide for translocation into the ER and are therefore not transported to lysosomes. In the case of cathepsin B, an alternate transcript lacking exons two and three is produced in various tissues (Mehtani et al. 1998). In addition to placing the translation start site within the propeptide region, this deletion leads to the generation of a mitochondrial import targeting sequence so that the newly synthesized protein product is located in that organelle (Müntener et al. 2004). The functional significance of the presence of the truncated proenzyme in this location and its status as a protease are still not clear. It has been shown that such a shortened propeptide reduces the folding competence of cathepsin B (Müntener et al. 2005). However, evidence has been provided that expression of truncated cathepsin B can provoke cell death in a manner independent of its enzymatic activity (Müntener et al. 2003; Baici et al. 2006).

Truncated procathepsin L gene products have also been reported. Here it was shown that initiation of translation occurs at methionine codons 3′ to the conventional initiation codon (Goulet et al. 2004). The translation product lacks the signal peptide and several residues of the propeptide. The resulting protein is cytoplasmic and the activity of such a product has been implicated in the processing of dynamin in proteinuric kidney disease (Sever et al. 2007). It has also been detected in the nucleus and is believed to process the transcription factor CPD/Cux (Goulet et al. 2006). However, it is still unclear whether proper disulfide bond formation and processing of the truncated proenzyme is possible in this cellular environment.

## 4.7 Biosynthesis and Molecular Forms of Cathepsin B

Cathepsin B usually occurs in human tissues and cell lines as a mixture of single-chain and double-chain variants. The ratio between the two forms can differ considerably between individual cell types. While conversion into the double-chain form is essentially complete in HepG2 cells (Mach et al. 1992), the single-chain enzyme is only partially processed in skin fibroblasts (Hanewinkel et al. 1987). In both cell types, cathepsin B is initially synthesized as a latent proenzyme of 45 kDa. Upon delivery to the lysosomal pathway, procathepsin B is first converted into the mature single-chain form of the proteinase (33 kDa). Further processing of this protein leads to the generation of the double-chain enzyme, consisting of subunits of 27 kDa (heavy chain) and 5 kDa (light chain). This endoproteolytic cleavage is accompanied by the excision of a dipeptide that connects the N-terminal light chain with the C-terminal heavy chain. The large fragment carries one *N*-glycan that is gradually lost due to the action of lysosomal glycosidases, ultimately giving rise to a carbohydrate-free, 24-kDa polypeptide (Mach et al. 1992). Furthermore,

cathepsin B is subject to exopeptidolytic trimming in the lysosomes, resulting in the removal of N- and C-terminal extensions each consisting of six amino acids. However, the extent of C-terminal cathepsin B processing varies between cell types. Removal of the C-terminal extension is quantitative in HepG2 cells (Mach et al. 1993), while C-terminal processing of cathepsin B is incomplete in human fibroblasts (Schmid et al. 1999).

In the case of cathepsin B, the subunits of the double-chain enzyme are held together by disulfide bridges. It has been shown that single-chain and double-chain forms are equivalent in their enzymatic properties. However, double-chain cathepsin B appears less stable than its single-chain counterpart (Hasnain et al. 1992). This is consistent with the hypothesis that intralysosomal generation of double-chain cathepsins represents a first step in the autocatalytic degradation of these proteinases (Erickson 1989).

Detailed information is also available on the proteolytic maturation of cathepsin B in rodent cells. In murine fibroblasts, cathepsin B is first synthesised as a 42-kDa precursor, which is then converted into the mature, single-chain enzyme (35 kDa). Further processing into the double-chain form of the proteinase is not observed. Therefore, at least two proteolytic steps occur during the biosynthesis of human fibroblast cathepsin B, while mouse procathepsin B is processed only at one site in these cells (Lorenzo et al. 2000; Probst et al. 2006). Similar results have been reported for rat hepatocytes, where single-chain cathepsin B also resists further proteolytic processing (Nishimura et al. 1988). However, a small amount of double-chain cathepsin B is generated during the biosynthesis of this enzyme in rat macrophages (Kominami et al. 1988).

### *4.7.1 Proteolytic Maturation of Cathepsin B: A Lysosomal Proteinase as Catalyst and Substrate*

In most mammalian cells, the generation of the mature single-chain forms of lysosomal proteinases including cathepsin B is believed to take place in acidic post-Golgi compartments. The local acidic environment in endosomes could be sufficient to trigger cathepsin B self-activation, as demonstrated for recombinant procathepsin B *in vitro* (Mach et al. 1994a). Presumably, activation is then quickly followed by both intra-molecular (Mach et al. 1994a; Quraishi and Storer 2001) and inter-molecular (Rowan et al. 1992; Rozman et al. 1999) autocatalytic cleavage events yielding mature single-chain cathepsin B. Terminal processing into the double-chain form is a much slower event and hence occurs most likely in lysosomes (Fig. 4.4).

In human and rodent cells, generation of double-chain cathepsin B can be abolished by treatment with synthetic cysteine proteinase inhibitors such as leupeptin, E-64d and Z-Phe-Ala-$CHN_2$ (Hanewinkel et al. 1987; Hara et al. 1988; Mach et al. 1992). This provided support for the proposal that processing of human

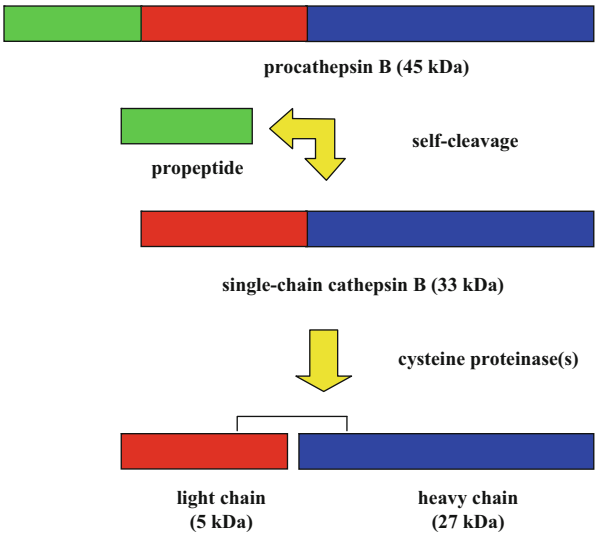


**Fig. 4.4** Proteolytic maturation of cathepsin B. The key steps in the proteolytic maturation of human cathepsin B are illustrated. Cathepsin B from other species is processed in an analogous manner. Cysteine proteinases of different families have been implicated in the intralysosomal generation of the double-chain form of the enzyme

cathepsin B in lysosomes is executed by cysteine cathepsins such as cathepsins B and L (Mach et al. 1992; Ishidoh and Kominami 2002). However, gene disruption studies have indicated that an unrelated cysteine proteinase, asparaginyl endopeptidase (legumain), plays a role in lysosomal processing of cathepsin B in murine kidney cells (Shirahama-Noda et al. 2003). Apart from internal fragmentation, cathepsin B appears to be also subject of processing by lysosomal exopeptidases (Mach et al. 1993). It has been proposed that cathepsin C accounts for the progressive trimming of three dipeptides from the N-terminus of the enzyme (Rowan et al. 1992), whereas the C-terminal extension of the proteinase can be removed by the dipeptidylcarboxypeptidase activity of cathepsin B itself (Rowan et al. 1993).

### *4.7.2 Biosynthesis of Cathepsin B in Cancer Cells*

Various investigators have reported the elevated synthesis and secretion of cathepsin B in response to viral transformation or malignant dedifferentiation (Sloane et al. 1981; Joyce et al. 2004). In the case of Moloney-murine-sarcoma-virus-transformed mouse fibroblasts, enhanced secretion of procathepsin B was attributed to the lack of functional M6P receptors on the cell surface (Achkar et al. 1990). The biosynthesis and molecular forms of cathepsin B have been studied in various human and rodent carcinoma cell lines. In most cases, no major differences to normal cells were detected (Mach et al. 1992; Braulke et al. 1992). However, unique glycoforms of cathepsin B as well as enhanced secretion of the proenzyme were observed in M6P/IGF2R-deficient SCC-VII murine squamous carcinoma

cells. Interestingly, SCC-VII cells lack mature dense lysosomes, with the bulk of intracellular acid hydrolases residing in immature granules with properties reminiscent of late endosomes (Lorenzo et al. 2000). Reconstitution of functional M6P/IGF2R expression in SCC-VII cells was sufficient to restore dense lysosome formation and cathepsin B retention (Probst et al. 2009). In contrast to SCC-VII cells, all other carcinoma cell lines studied so far were found to be M6P/IGF2R-positive. Accordingly, these tumour cells secrete normal amounts of procathepsin B (Mach et al. 1992; Braulke et al. 1992). Interestingly, this also applies to SW1116 human colon carcinoma cells which are unable to internalise M6P-modified proteins despite normal M6P/IGF2R expression (Braulke et al. 1992). These findings suggest that receptor-mediated endocytosis of secreted procathepsin B does not contribute significantly to lysosomal sorting of the enzyme in tumour cells.

### *4.7.3 Extracellular Forms of Cathepsin B and the Mechanisms of Its Release and Activation*

Under normal conditions, lysosomal proteinases occur in the pericellular environment only as their latent precursors. However, enzymatically active extracellular forms of cathepsin B have been found in tumours, either in a soluble state (Poole et al. 1978) or bound to the plasma membrane (Sloane et al. 1986). Possible explanations for these phenomena include autoactivation of secreted latent procathepsin B and/or regulated exocytosis of the mature enzyme from lysosomes. It has been demonstrated that secreted procathepsin B may undergo self-activation triggered by the acidic microenvironment around tumour cells, leading to the transient formation of non-covalent complexes between cathepsin B and its autoinhibitory propeptide and thus stabilisation of the mature enzyme which would be otherwise short-lived in body fluids (Mach et al. 1994b). Interestingly, it has been observed that self-activation of procathepsin B is accelerated in the presence of negatively charged macromolecules like glycosaminoglycan chains, which are major constituents of the extracellular matrix (Mach et al. 1994a). Recently, evidence has been provided that the proregion accounts for the interaction of the cathepsin B precursor with negatively charged molecules as found on cellular surfaces (Caglic et al. 2007). Cathepsin B has been detected at the plasma membrane of transformed breast epithelial cells and colon carcinoma cells (Sloane et al. 1994; Cavallo-Medved et al. 2005). In addition, procathepsin B was found to interact with the small subunit of the peripheral membrane protein annexin II, which could contribute to the presence of cathepsin B on the surface of tumour cells (Mai et al. 2000).

An alternative hypothesis to explain the extracellular occurrence of enzymatically active cathepsin B in tumours relies on the observation that this enzyme is frequently redistributed to peripheral vesicles in tumour cells (Rozhin et al. 1994). It was proposed that this could promote exocytosis of cathepsin B due to retrograde

transport from lysosomes along the endocytic pathway followed by fusion of endosomal compartments with the plasma membrane. Such processes also occur under physiological conditions, as for instance in the case of the degranulation of the highly specialised secretory lysosomes of lymphocytes (Blott and Griffiths 2002). However, other cell types are also able to secrete their lysosomal contents. For instance, such a regulated release of cysteine cathepsins seems to occur in the process of the generation of thyroid hormones. It was observed that thyroid epithelial cells secrete mature cathepsin B, thus initiating limited extracellular proteolysis of the hormone precursor thyroglobulin which ultimately culminates in the generation of the thyroid hormone thyroxine (Friedrichs et al. 2003). It was shown that regulated secretion of cathepsin B by thyroid epithelial cells is linked to the redistribution of cathepsin B-containing vesicles from the perinuclear region to the cell periphery, indicating that the enzyme is first delivered to endosomal/lysosomal compartments and then secreted (Linke et al. 2002).

## 4.8 Biosynthesis of Cathepsin L

Cathepsin L was initially reported to be 'the most active endopeptidase from rat liver lysosomes acting at pH 6–7' (Kirschke et al. 1977). Later, a protein secreted by various transformed cells (Gottesman 1978) was determined to be procathepsin L (Gal and Gottesman 1986; Joseph et al. 1988), suggesting that the physiological functions of this cathepsin are not limited to general protein turnover within lysosomes. A cDNA cloned from mouse macrophages (Portnoy et al. 1986) was predicted to encode procathepsin L based on alignment with sequences of human liver cathepsin L (Mason et al. 1986). The cDNA of the rat enzyme was sequenced a year later (Ishidoh et al. 1987), and the sequence of the cDNA encoding the human enzyme was reported in 1988 (Gal and Gottesman 1988; Joseph et al. 1988). The first crystal structure of human procathepsin L (Fig. 4.1) was reported in 1996 (Coulombe et al. 1996).

Procathepsin L was initially predicted to reach lysosomes via the classic secretory pathway based on the fact that the enzyme possesses high-mannose *N*-linked oligosaccharides. Thus the presence of a 17-amino acid signal peptide mediating ER import was predicted (Portnoy et al. 1986) and subsequently confirmed experimentally by radiosequence analysis of immunoprecipitated proenzyme (Erickson 1989). Disc electrophoretograms of the purified enzyme first identified multiple forms of cathepsin L in rat liver (Kirschke et al. 1977), suggesting that enzyme maturation requires post-translational proteolysis. This was confirmed and the relationship of the various cathepsin L isoforms was established by pulse-chase analysis of the mouse enzyme (Gal et al. 1985; Portnoy et al. 1986). Preprocathepsin L from all species loses a signal peptide co-translationally, an activation peptide in late endosomes, and undergoes chain cleavage in lysosomes (Fig. 4.5a).

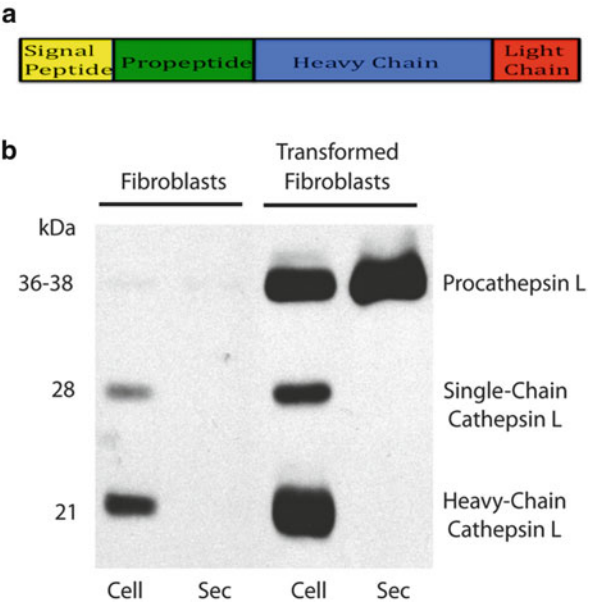


**Fig. 4.5** Procathepsin L synthesis, storage and secretion are increased in transformed fibroblasts. (**a**) Diagram of preprocathepsin L. The N-terminal signal peptide is cleaved off co-translationally, while the propeptide is removed to activate the enzyme once the protein reaches late endosomes. In late endosomes or lysosomes single-chain cathepsin L is cleaved into a heavy and a light chain held together by a disulfide bridge. (**b**) These biosynthetic forms of cathepsin L can be visualized in Kirsten virus-transformed KNIH mouse fibroblasts and wild-type mouse fibroblasts incubated in serum-free medium for 2 h. Equal amounts of cellular protein (Cell) and proteins secreted to the culture medium (Sec) were resolved by polyacrylamide gel electrophoresis and visualized by western blotting. Cell transformation results in increased synthesis of cathepsin L, increased secretion of the proenzyme, and cellular storage of the proenzyme. The majority of the cathepsin L protein synthesized by the KNIH cells (46 %) is present in the 2 h culture medium as the 38-kDa proform of the protease. Significant procathepsin L remains in the transformed fibroblasts (23 %), avoiding activation in lysosomes. Figure published in Traffic 1: 724–737 (2000)

Co-translationally, before synthesis of the protein is complete, high-mannose carbohydrate chains are added to one and sometimes two selected asparagine residues of procathepsin L. For the mouse proenzyme, the sugar chain is added at Asn221 (numbering from the first residue of the signal peptide). Mouse Asn268 is normally not modified, but high expression levels resulting in chaperone shortage and thus slow folding, point mutations in the propeptide, or addition of a C-terminal epitope tag can modify the conformation sufficiently to expose this site to oligosaccharyltransferase (Chapman et al. 1997). Glycosylation of cathepsin L is not essential for enzymatic function and has little effect on protein folding or stability (Smith et al. 1989). Procathepsin L that lacks carbohydrate and thus M6P residues is secreted (Kane 1993; Smith et al. 1989). Procathepsin L that acquires two high-mannose carbohydrate chains subsequently undergoes modification with complex sugars, which are detected by increased molecular mass and resistance of the sugar

chain to endoglycosidase H (Chapman et al. 1997; Collette et al. 2004a). This form of the protease is also rapidly secreted and is not detectable within cells unless a reagent such as brefeldin A is utilized to block constitutive secretion (Chapman et al. 1997).

Modification of the high-mannose carbohydrate chain(s) on lysosomal proteases with phosphate is critical for segregation out of the secretory pathway. For mouse cathepsin L, the key residues for recognition by phosphotransferase are Lys54 and Lys99, but low levels of M6P modification can still be detected when these residues are ablated, indicating that other lysines can substitute (Cuozzo et al. 1998; Warner et al. 2002). The oligosaccharides bound to procathepsin L are thought to be uniformly processed to diphosphorylated species (Lazzarino and Gabel 1990; Stearns et al. 1990). As correctly folded procathepsin L has only a single carbohydrate chain and as phosphorylated high-mannose oligosaccharides cannot be converted into complex *N*-glycans (Kornfeld and Kornfeld 1985), procathepsin L remains sensitive to endoglycosidase H. The M6P recognition marker generated by phosphotransferase recognition of the patch of charged lysine residues is bound by one of the two M6P receptors, M6P/IGF2R or MPR46. As procathepsin L has only a single phosphorylated oligosaccharide, its affinity for M6P/IGF2R is low relative to other lysosomal enzymes (Dong and Sahagian 1990). Additionally, uncharacterized protein determinants are thought to impair its interaction with M6P/IGF2R (Lazzarino and Gabel 1990).

### *4.8.1 Proteolytic Processing and Activation of Procathepsin L*

Most cysteine cathepsin precursors contain an autoinhibitory propeptide to avoid premature enzymatic activity during intracellular targeting. As for other cathepsins, it is believed that proteolytic removal of the cathepsin L propeptide/activation peptide only occurs once the protein reaches the acidic endolysosomal membrane system. Pulse-chase analysis in mouse fibroblasts (Gal et al. 1985) and mouse macrophages (Portnoy et al. 1986) confirmed that a propeptide is removed from a 36–38 kDa proenzyme precursor (Fig. 4.5b). The site of the cleavage that releases the 96-amino-acid mouse macrophage propeptide was determined by radiosequencing of immunoprecipitated protein (Erickson 1989). The cleavage site can vary, however, suggesting that either multiple sites are utilized and/or that exopeptidase cleavage occurs. Intermediates have been detected (Ishidoh and Kominami 1994; Ishidoh et al. 1998; Ménard et al. 1998; Salminen and Gottesman 1990), but these are not necessarily fully proteolytically active (Ishidoh and Kominami 2002). Structural studies of the procathepsin L propeptide show that the N-terminal portion of the propeptide occludes the active site (Carmona et al. 1996), while the C-terminal portion serves as a chaperone that initiates correct folding of the protein within the ER (Schilling et al. 2001; Tao et al. 1994). The propeptide avoids degradation, although positioned in the active site, because it lies in the pocket in opposite orientation to cleavable substrates (Coulombe et al. 1996).

Procathepsin L can autoactivate *in vitro* (Mason et al. 1987; Ménard et al. 1998) and *in vivo* (Nomura and Fujisawa 1997; Salminen and Gottesman 1990) if the pH drops below 5. Self-activation can also occur at higher pH (5.5–6.0) if the proenzyme is in the presence of a negatively charged surface, such as that presented by dextran sulfate (Mason and Massey 1992) or glycosaminoglycans, e.g. those of heparan sulfate or chondroitin sulfate proteoglycans (Ishidoh and Kominami 1995; Kihara et al. 2002). Cathepsin D has also been shown to be capable of activating procathepsin L (Nishimura et al. 1989; Wiederanders and Kirschke 1989). The activation of procathepsin L is a regulated event; thus the amount of proenzyme in cells varies with the cell type and its physiological state (Fig. 4.5b). While the proenzyme is barely detectable in normal fibroblasts, it is commonly the major form of cathepsin L in transformed fibroblasts (Ahn et al. 2002; Collette et al. 2004a). Thus, measuring enzyme activity alone does not reveal the total amount of the protease in cells, while assaying mRNA levels alone does not provide a reliable measure of active cathepsin L levels present in cells or tissues.

Once the enzyme reaches acidic compartments, additional proteolysis leads to generation of light and heavy cathepsin L chains. As initially detected by pulse-chase analysis, conversion of the mouse 36–38 kDa proform to a 28-kDa single-chain enzyme is followed by cleavage to a 21-kDa heavy chain derived from the N-terminus of the single chain and a 6–7 kDa light chain derived from the C-terminus of the single chain (Fig. 4.5a). The small light chain is not routinely detected on standard polyacrylamide gels (Gal et al. 1985; Portnoy et al. 1986). The sites of cleavage in human and rat cathepsin L have been determined by sequencing of the purified double-chain enzymes, which revealed that 2–3 amino acids are missing at the light-heavy chain boundary due to exopeptidase cleavage (Mason et al. 1986; Ishidoh et al. 1987; Towatari and Katunuma 1988; Ritonja et al. 1988). Recently, asparaginyl endopeptidase has been implicated in double-chain cathepsin L formation as mouse cells lacking the former enzyme accumulate the single-chain form (Shirahama-Noda et al. 2003; Maehr et al. 2005). However, inhibitor studies have also provided evidence for the involvement of cysteine cathepsins, including cathepsin L itself, in this process (Hara et al. 1988; Salminen and Gottesman 1990; Nishimura et al. 1995; Ahn et al. 2002). The two chains remain connected through a disulfide bridge. Active-site labelling experiments established that both the single and the double-chain forms of the enzyme are proteolytically active (Mason et al. 1989). Like removal of the activation peptide, the efficiency of this secondary cleavage depends on the cell type. The amount of heavy chain detected in cell extracts relative to the single-chain form is thus variable (Erickson 1989).

### *4.8.2 Non-lysosomal Localization of Cathepsin L*

Cathepsin L activity has been detected at multiple intra- and extracellular sites, suggesting the protease has specific physiological functions in addition to general protein turnover in lysosomes. In certain cell types, enzyme activity is found in

endosomes, in secretion granules and/or secretory lysosomes, in the cytoplasm, and in the nucleus. Thus, targeting mechanisms must exist to mediate transport to sites other than lysosomes.

An initial indication that procathepsin L can function extracellularly, contributing to cancer cell metastasis, came from the observation that transformed mouse fibroblasts secrete large quantities of a protein (Fig. 4.5b) initially named the 'major excreted polypeptide' or MEP (Gottesman 1978). Expression of this protein is induced by oncogenic Ras (Joseph et al. 1987). MEP was shown to be modified with M6P moieties (Sahagian and Gottesman 1982), localized to lysosomes (Gal et al. 1985), and eventually found to be identical to procathepsin L (Joseph et al. 1987; Mason et al. 1987; Troen et al. 1987). Support for the idea that secreted cathepsin L might serve physiological functions extracellularly came from the demonstration that MEP could degrade extracellular matrix (Gal and Gottesman 1986), and that purified enzyme could efficiently degrade kidney glomerular basement membrane (Baricos et al. 1988). Additional evidence that cathepsin L can serve a physiological function outside cells comes, for example, from demonstration that secreted cathepsin L can liberate thyroid hormone (Brix et al. 1996) and from the extensive studies of cathepsin L-like proteases secreted by parasites (Robinson et al. 2008).

Most lysosomal proteases are constitutively secreted to some minor extent, presumably because of failure to bind to M6P receptors in the TGN, but relative to procathepsin L, other endogenous lysosomal enzymes are efficiently targeted to lysosomes in transformed fibroblasts. Thus this secretion is selective for procathepsin L. The upregulation of gene expression that is characteristic of transformation (Ishidoh and Kominami 1998) would lead to increased synthesis of protein that could swamp M6P receptors, causing the excess proenzyme to be secreted by the constitutive secretory pathway. This, however, should concomitantly result in increased secretion of other lysosomal enzymes synthesized at lower levels, which must compete for binding to M6P receptors; surprisingly, this is not detected. Consistent with this, while ectopic expression of Ras leads to increased expression of procathepsin L in both fibroblasts and epithelial cells, high secretion is only detected in fibroblasts, which secrete 50 % of their total cathepsin L, compared to 16 % for epithelial cells (Collette et al. 2004b). Recently, procathepsin L secretion by human melanoma cells has been correlated with Rab4 expression and/or function (Barbarin and Frade 2011). However, the exact nature of the molecular mechanisms underlying these unique properties of procathepsin L within the secretory pathway remains unknown.

The primary form of cathepsin L detected outside transformed fibroblasts is the proteolytically inactive proenzyme, leading to questions as to the physiological relevance of the secreted protease, as the extracellular pH is normally assumed to be neutral and cathepsin L has been reported to be the most unstable of the lysosomal cysteine proteases at neutral or alkaline pH (Turk et al. 1993). The demonstration that extracellular matrix-like molecules rich in negative charge can induce

self-activation provides a mechanism for activation of the secreted protease (Ishidoh and Kominami 1995; Kihara et al. 2002). Additionally, numerous studies have established that the pericellular pH of tumour cells can be sufficiently low to enable cathepsins to be active (Stubbs et al. 2000). Finally, the activity of cathepsin L has been shown to be modulated by the extracellular matrix proteoglycan testican-1 (Bocock et al. 2003) and by purified thyroglobulin type-1 domain (Meh et al. 2005) found not only in secreted proteins such as thyroglobulin (Brix et al. 1996) and testican-1, but also in the p41 isoform of the invariant chain associated with MHCII molecules (Bevec et al. 1996; Hitzel et al. 2000). The p41 protein not only stabilizes mature cathepsin L in endocytic compartments of antigen-processing endosomes (Turk et al. 1999), but like testican-1 (Bocock et al. 2003), stabilizes active cathepsin L in the neutral extracellular environment, possibly potentiating its role in the inflammatory response (Fiebiger et al. 2002). Together these observations argue that the secreted proenzyme can impact extracellular events.

Active, mature cathepsin L can also be detected in cell culture medium under certain physiological conditions. Activated thioglycollate-elicited mouse macrophages abundantly secrete single-chain and double-chain forms of cathepsin L, in contrast to macrophages resident in the peritoneum, which primarily secrete proenzyme (Erickson 1989; Collette et al. 2004a). The cellular pathway mediating secretion of active cathepsin L has not been elucidated but may relate to the recent finding that almost all proteins in human primary macrophages lack M6P and thus utilize an M6P-independent pathway for targeting proteases to lysosomes (Pohl et al. 2010).

### *4.8.3 M6P-Independent Intracellular Transport of Cathepsin L*

Early evidence that cathepsin L could be packaged in regulated secretory vesicles came from the detection of the protease in sperm acrosomes (McDonald and Kadkhodayan 1988) and in melanosomes (Diment et al. 1995). Cathepsin L has more recently been documented to cleave perforin in cytotoxic granules of natural killer cells and cytotoxic T lymphocytes (Konjar et al. 2010) and to participate in neuropeptide production in neuroendocrine cells (Yasothornsrikul et al. 2003). Incorporation into vesicles capable of regulated secretion allows directed delivery to specific sites on the membrane, such as an immunological synapse (Griffiths et al. 2010), while constitutive secretion from the TGN would deliver the protease to basolateral surfaces. These observations suggest that, at least in certain cell types, a mechanism exists to target procathepsin L to secretory granules, as well as to lysosomes.

Procathepsin L has been detected in dense-core vesicles of transformed mouse fibroblasts (Yeyeodu et al. 2000; Ahn et al. 2002), suggesting that the protease can

be stored in regulated secretory vesicles even in a non-secretory cell type (Fig. 4.6). The use of an antibody specific for the propeptide established that it is the latent proenzyme that is concentrated in these vesicles, explaining the stability of the large amount of proenzyme detectable in these cells under steady-state conditions (Yeyeodu et al. 2000; Ahn et al. 2002), in contrast to untransformed fibroblasts such as NIH3T3 or L fibroblasts which contain primarily mature single-chain and double-chain cathepsin L (Fig. 4.5b). Colocalization with the transmembrane protein CD63 (Ahn et al. 2002) indicates that this site of storage is a multivesicular endosome, an endosome that can undergo fusion with the plasma membrane in response to signaling (Piper and Katzmann 2007). This is consistent with the recent demonstration that fibroblasts contain specialized endosomes which fuse with the plasma membrane in response to changes in cytosolic calcium levels (Laulagnier et al. 2011).

Procathepsin L has been observed to undergo self-association in yeast two-hybrid assays (Yeyeodu et al. 2000), suggesting that high synthesis levels could lead to formation of procathepsin L aggregates, possibly as early as in the ER. These aggregates might react less efficiently with M6P receptors than the monomeric proenzyme. The low affinity of procathepsin L for M6P/IGF2R (Dong and Sahagian 1990; Lazzarino and Gabel 1990) may also contribute to storage granule targeting. Alternatively, an unidentified alternate targeting receptor which could mediate the transport of these proteases to secretory vesicles could be selectively expressed or upregulated upon cell transformation.

The existence of M6P-independent or alternate targeting pathways for lysosomal proteins has been suggested since the early observation that I-cell hepatocytes, Kupffer cells and leukocytes that lack phosphotransferase activity possess functional lysosomes (Owada and Neufeld 1982; Waheed et al. 1982). Sortilin has recently been reported to bind several lysosomal enzymes, explaining their M6P-independent targeting to lysosomes in I-cell fibroblasts, but cathepsin L reaches lysosomes in these cells without binding sortilin (Canuel et al. 2009), suggesting the existence of additional, yet unidentified targeting receptor(s). Procathepsin L, but not the active protease, has been observed to undergo pH-dependent M6P-independent association with a 43-kDa mouse fibroblast membrane protein (McIntyre and Erickson 1991, 1993), an interaction inhibited by a 9-residue fragment of the N-terminal activation peptide (McIntyre et al. 1994). Procathepsin D undergoes similar M6P-independent membrane association in these and other cells (McIntyre and Erickson 1991; Zhu and Conner 1994; Godbold et al. 1998). A receptor mediating this interaction has not been isolated so far. Precedence for M6P-independent, concentration-dependent sorting of proteases to storage vesicles comes from studies of plant cells that contain protein storage vacuoles, which likewise store enzymes in dense cores comprised of internal membranes (Wang et al. 2011). Protein targeting to these vacuoles is mediated by 43-kDa homology-transmembrane RING-H2 (RMR) proteins (Jiang et al. 2000),

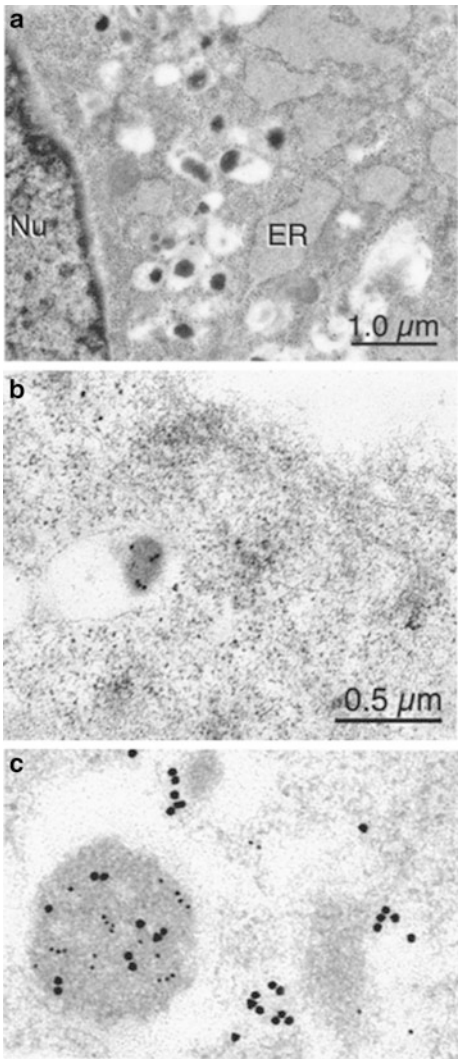


**Fig. 4.6** Procathepsin L of transformed fibroblasts is stored in dense cores of multivesicular endosomes. Procathepsin L was localized in Kirsten virus-transformed KNIH mouse fibroblasts (Panel **a**) using polyclonal rabbit antibodies specific for the 96-amino acid propeptide of mouse procathepsin L detected with goat anti-rabbit Fab fragments conjugated with 15-nm gold (Panel **b**). In Panel (**c**), the multivesicular endosome marker protein CD63 was colocalized in the same section using guinea pig anti-rat CD63 antibodies that were detected with biotinylated goat anti-guinea pig IgG, followed by avidin conjugated to 20-nm gold. Procathepsin L was detected with antibodies specific for the propeptide followed by goat anti-rabbit IgG conjugated with 10-nm gold. Figure published in Traffic 3: 147–159 (2002). *ER* endoplasmic reticulum, *Nu* nucleus

integral endosomal membrane PA-TM-RING proteins (Erickson 2011) which have an N-terminal protease-associated (PA) domain that recognizes C-terminal sequences on ligands (Wang et al. 2011).

## 4.9 Biosynthesis of Other Cysteine Cathepsins

Cathepsin H is one of the two other cysteine cathepsins whose biosynthesis and intracellular transport has been thoroughly characterized. This enzyme is synthesized in rat hepatocytes and rat macrophages as a 41-kDa proenzyme.

The latent precursor is rapidly converted into the mature single-chain form of the proteinase (28 kDa). A double-chain form of cathepsin H composed of an N-terminal large subunit (22 kDa) and a C-terminal light chain (6 kDa) is generated in macrophages (Kominami et al. 1988), while single-chain cathepsin H persists in hepatocytes even upon prolonged incubation (Nishimura et al. 1988). Generation of the double-chain form can be inhibited by treatment with the cysteine cathepsin inhibitor E-64d (Hara et al. 1988). Interestingly, genetic ablation of asparaginyl endopeptidase expression exerts a similar inhibitory effect on cathepsin H processing in mice (Shirahama-Noda et al. 2003). In contrast to cathepsin B, mature cathepsin H is not subject of N- and C-terminal trimming by exopeptidases. A glycosylated octapeptide derived from the prosegment is linked to the mature enzyme through a disulfide bridge, binding to the active-site cleft of the proteinase in a substrate-like manner. Cathepsin H is also *N*-glycosylated within its catalytic domain, carrying high-mannose *N*-glycans as typical for soluble lysosomal proteins. Evidence has been provided that the presence of these oligosaccharides is required for the delivery of the enzyme to lysosomes (Nishimura et al. 1988). Recently, it has been shown that cathepsin H binds to M6P/IGF2R in an M6P-dependent manner (Sleat et al. 2006). These findings indicate that cathepsin H can be transported to lysosomes via the M6P receptor pathway. However, a recent study has highlighted that lysosomal targeting of cathepsin H could also involve interactions with alternate sorting receptors such as sortilin (Canuel et al. 2008).

The biosynthesis and intracellular transport of cathepsin C has been studied in rat hepatocytes and hepatoma cells. This oligomeric enzyme is first synthesized as a precursor with an apparent molecular mass of 55 kDa. Procathepsin C is *N*-glycosylated and carries the M6P recognition marker in its carbohydrate moiety. Transport to lysosomes has been shown to depend on its interaction with M6P receptors (Mainferme et al. 1985; Muno et al. 1993). Upon delivery to lysosomes, the cathepsin C precursor is proteolytically processed into 25-kDa and 8-kDa fragments, corresponding to the heavy and light chains of the mature enzyme (Ishidoh et al. 1991). In contrast to cathepsins B and L, procathepsin C maturation is not affected by treatment of the cells with the cysteine proteinase inhibitor Z-Phe-Ala-$CHN_2$ (Mainferme et al. 1985).

Comparatively little information is available on the biosynthesis and intracellular transport of other cysteine cathepsins. Evidence has been provided that procathepsins F, K, S and X are modified with M6P residues (Czupalla et al. 2006), and their mature counterparts have been localized in lysosomes (Wiederanders et al. 1992; Tepel et al. 2000; Kos et al. 2005; Tang et al. 2006; van Meel et al. 2011). None of these cathepsins seems to undergo processing into a double-chain species. Two variants of mature cathepsin V have been detected in lysosomal fractions of human thyroid carcinoma cells (Tedelind et al. 2010), which suggests intralysosomal conversion of the single-chain enzyme into a double-chain form as in the case of its close relative cathepsin L. Opposite to all other cysteine cathepsins, the precursor of cathepsin W is retained in the endoplasmic reticulum

and appears to be resistant to proteolytic maturation (Wex et al. 2001; Ondr and Pham 2004).

## 4.10 Biosynthesis and Intracellular Transport of Cathepsin D

The major non-cysteine cathepsin is the aspartyl proteinase cathepsin D. The open reading frame of human cathepsin D cDNA (Faust et al. 1985) encodes a polypeptide of 412 amino acids with N-terminal pre- and prosegments of 20 and 44 residues, as judged by comparison with the N-terminal sequences of the biosynthetic precursors of porcine cathepsin D (Erickson and Blobel 1979; Erickson et al. 1981). From a structural point of view, mature human cathepsin D exists as a single-chain protein, which is relatively more abundant in endosomes, and as a double-chain form, which is relatively more abundant in lysosomes (Hasilik and Neufeld 1980; Follo et al. 2007). The initial product of its biosynthesis is preprocathepsin D, which upon entry into the lumen of the rough ER loses its signal peptide, leading to the formation of procathepsin D consisting of 392 amino acids. While synthesis is still ongoing, procathepsin D is *N*-glycosylated on both Asn134 and Asn263, belonging to the N-terminal and C-terminal lobes, respectively, in the double-chain mature polypeptide (Faust et al. 1985). These Asn residues are located in a peptide region specifically involved in the recognition of cathepsin D by phosphotransferase (Baranski et al. 1990, 1991, 1992; Cantor et al. 1992; Dustin et al. 1995) and thus can be decorated with *N*-linked oligosaccharides harbouring M6P residues. The last step of M6P biosynthesis occurs in a compartment beyond the site of action of brefeldin A (Radons et al. 1990), a fungal antibiotic that disrupts the organization of the Golgi stacks and causes retrograde transport of Golgi proteins to the ER (Lippincott-Schwartz et al. 1989). The facts that ammonium chloride impairs the uncovering of phosphorylated procathepsin D and stimulates the secretion of procathepsin D bearing masked M6P residues (Isidoro et al. 1990) further support the view that the two reactions are spatially separated, in agreement with the different subcellular localizations of the two enzymes involved in the generation of the M6P recognition marker (Rohrer and Kornfeld 2001; Tiede et al. 2005).

The molecular weight of doubly glycosylated procathepsin D is about 53 kDa. Procathepsin D is then entrapped within vesicles budding from the TGN and transported to the endosomal compartments by M6P-dependent and M6P-independent routes. In the TGN, M6P receptors sequester procathepsin D into transport vesicles that travel along microtubules and then fuse with pre-lysosomal organelles, whereupon procathepsin D is discharged and activated due to the mild acidic milieu of these compartments. Both M6P/IGF2R and MPR46 are involved in this transport (Braulke and Bonifacino 2009), though the former receptor exhibits a greater affinity for procathepsin D (Pohlmann et al. 1995).

Nonetheless, procathepsin D devoid of M6P residues may reach endosomal compartments (Glickman and Kornfeld 1993) by an M6P-independent route that possibly involves the transient interaction with the lysosomal protein prosaposin (Zhu and Conner 1994; Gopalakrishnan et al. 2004). Very recently, it has been shown that transport of procathepsin D to lysosomes at least partially depends on its interaction with sortilin (Canuel et al. 2008).

Once procathepsin D is released into the lumen of late endosomes, the propeptide segment of 44 amino acids is proteolytically removed and an enzymatically active, single-chain enzyme is formed (Fig. 4.7). This molecular form has a molecular weight of about 48 kDa and consists of 348 amino acids. The removal of the propeptide is not purely an autoproteolytic process, as it occurs also in a mutant form (D295N) of human procathepsin D in which the active site of the enzyme has been inactivated. Rather, cysteine cathepsins such as cathepsins B and L are probably involved in this processing event (Laurent-Matha et al. 2006). The predominant molecular form of human cathepsin D is in fact the mature double-chain protein. It is composed of an N-terminal light chain of 14 kDa and a C-terminal heavy chain of 34 kDa (Hasilik and Neufeld 1980; Gieselmann et al. 1983). The light and heavy chains of cathepsin D are not covalently linked by inter-chain disulfide bonds, as previously believed; rather, their association arises from hydrophobic and steric interactions between the two chains, which keep their proper conformations due to intra-chain disulfide bridges. The conversion of single-chain cathepsin D into the double-chain form is a lysosomal event and depends on the activity of cysteine cathepsins (Gieselmann et al. 1985; Samarel et al. 1989). Cathepsin D accumulating in lysosomes undergoes progressive C-terminal trimming that removes the two residues Leu412 and Arg411, and probably others, from the heavy chain. The trimming at the heavy-chain C-terminus was first shown for the double-chain form of porcine cathepsin D (Erickson and Blobel 1983). It has been proposed that this late processing initiates the turnover of cathepsin D (Erickson 1989).

### *4.10.1 Biosynthesis of Rodent Cathepsin D*

Purified rat liver cathepsin D is a mixture of a single-chain form (approximately 95 %) and two different double-chain proteins. The rat cathepsin D cDNA encodes a polypeptide containing a 20-residue signal sequence followed by a 44-residue propeptide (Fujita et al. 1991). A plethora of molecular forms of rat cathepsin D, comprising the 53 kDa precursor, the 47 kDa intermediate, the 43 kDa mature single-chain proteinase, two 34/30 kDa heavy chains and two 9/14 kDa light chains has been described in rat basophilic leukemia cells (Dragonetti et al. 2000). This study confirmed that the most abundant rat cathepsin D form is the mature single-chain enzyme, and revealed that the predominant double-chain isoform is composed of the 34-kDa and 9-kDa chains.

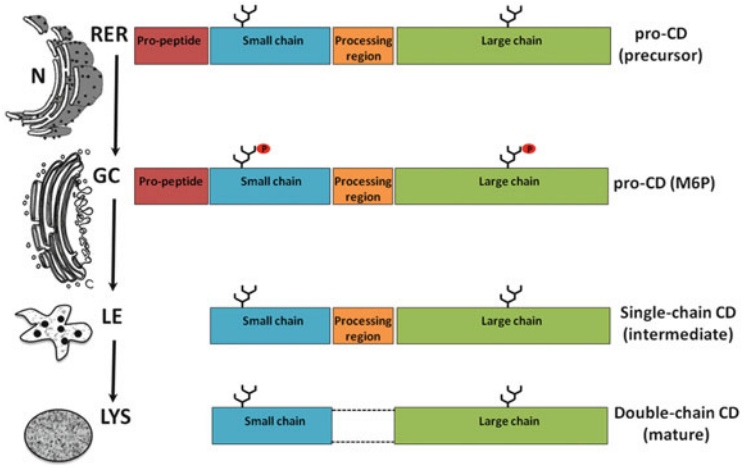


**Fig. 4.7** Proteolytic maturation of cathepsin D. The key steps in the proteolytic maturation of human cathepsin D (CD) are illustrated. The intralysosomal generation of the double-chain form of the enzyme does not occur in all species. *GC* Golgi complex, *LE* late endosome, *LYS* lysosome, *N* nucleus, *RER* rough endoplasmic reticulum

The complete cDNA sequence coding for mouse cathepsin D has also been reported (Grusby et al. 1990). The molecular forms of cathepsin D found in mouse 3T3 fibroblasts include the precursor (with a molecular weight of about 52 kDa) and mature polypeptides mostly represented by a 48 kDa single-chain isoform and, to a minor extent, by a double-chain protein composed of 31-kDa and 16-kDa heavy and light chains (Isidoro et al. 1995). Rat and mouse cathepsin D both contain two potential *N*-glycosylation sites, one in each chain.

### *4.10.2 Biosynthesis and Molecular Forms of Cathepsin D in Non-mammalian Species*

Chicken (*Gallus gallus*) cathepsin D has been purified from oocytic yolk, pre-ovulatory follicle and liver homogenates by affinity chromatography. Based on peptide sequencing of the N-terminus, a full-length cDNA clone encoding chicken cathepsin D was isolated from a chicken follicle cDNA library. It is predicted that chicken cathepsin D exists as a double-chain isoform (Retzek et al. 1992). In 1997, Gerhartz and colleagues purified and characterized cathepsin D from the yolk-sac membrane of quail (*Coturnix coturnix japonica*) eggs. The molecular forms of mature quail cathepsin D include a double-chain isoform composed of a light chain of 14 kDa and a heavy chain of 30 kDa, which is the most abundant molecular species, and a single-chain isoform of about 44 kDa

(Gerhartz et al. 1997). The entire amino-acid sequence of quail cathepsin D is not known, and its cDNA has not been cloned yet.

The molecular features of cathepsin D isolated from *Chionodraco hamatus* (Antarctic icefish) and *Clupaea harengus* (Atlantic herring) are known. The mature enzyme purified from the liver of Antarctic icefish shows a molecular weight of 40 kDa (Capasso et al. 1999), whereas that isolated from herring muscle shows a molecular weight of 38–39 kDa (Nielsen and Nielsen 2001). In both fishes cathepsin D has been found to occur exclusively as a single-chain isoform. The N-termini of icefish and herring cathepsin D have been determined by automated Edman degradation (Capasso et al. 1999; Nielsen and Nielsen 2001). No data are available about the glycosylation status of icefish cathepsin D. Glycosidase treatments (with endoglycosidase H or *N*-glycosidase F) and binding to the lectin concanavalin A indicate that herring cathepsin D contains only one *N*-linked carbohydrate moiety of the high-mannose type (Nielsen and Nielsen 2001). Very recently, the biosynthesis of *Danio rerio* (zebrafish) cathepsin D has been characterized. The protein is synthesized as a mono-glycosylated precursor of 43 kDa that is transported in an M6P-dependent manner to endosomal-lysosomal compartments where it matures and accumulates as a single-chain peptide of 41 kDa (Follo et al. 2011).

*Xenopus laevis* is a frog commonly used as a model organism for studies on embryonic development. Mature cathepsin D of *Xenopus laevis* shows different isoforms, depending on the examined tissue: in fact, two different isoforms of 43 and 36 kDa can be isolated from ovarian extracts, while only the 36-kDa isoform is present in liver extracts (Nakamura et al. 1996).

*Aedes aegypti* is a mosquito that can spread the Dengue and Yellow fever viruses. A cDNA coding for a mosquito cathepsin D-like lysosomal aspartic proteinase was cloned and sequenced. The cDNA encodes a polypeptide of 387 amino acids starting with an 18-residue signal sequence and a 35-residue pro-segment. Mosquito cathepsin D purified by pepstatin A-agarose displayed an apparent molecular weight of 80 kDa or 40 kDa under non-denaturing or denaturing conditions, respectively. Two-dimensional gel electrophoresis revealed a predominant spot of 40 kDa with an isoelectric point of 5.4. The mature enzyme is a single-chain polypeptide with only one *N*-glycosylation site in the N-terminal part of the protein (Cho and Raikhel 1992). Cathepsin D cDNA from the silkworm *Bombyx mori* was cloned in 2006 (Gui et al. 2006) and encodes a single-chain protein of 385 amino acids bearing one potential *N*-glycosylation site, with an apparent molecular weight of 40–44 kDa. *B. mori* cathepsin D contains the two active-site aspartic acid residues as well as the six cysteine residues characteristic of aspartic proteinases, and displays substantial amino-acid identity with the aspartic proteinases of the mosquitoes *Anopheles gambiae* (64 % identity) and *Aedes aegypti* (63 % identity). Three cDNAs coding for preprocathepsin D-like proteinases have been cloned from *Musca domestica*, but these await further characterization on the protein level (Padilha et al. 2009).

*Caenorhabditis elegans* is a small unsegmented nematode. Cathepsin D purified by affinity chromatography from this worm is a mixture of several enzymatically active single-chain isoforms, which differ in their glycosylation status, with an

apparent molecular weight of 36 and 34 kDa (the most abundant forms) and of 32, 38 and 44 kDa (Jacobson et al. 1988). Cathepsin D has been also isolated from the trematode *Schistosoma japonicum* (commonly known as blood fluke). The precursor and the active single-chain enzyme showed an apparent molecular weight of 47 kDa and 41 kDa, respectively (Verity et al. 1999).

The slime mold *Dictyostelium discoideum* is a eukaryotic amoeba phylogenetically very distant from man. Slime mold cathepsin D, Ddp 44, was first identified in 1999 by peptide sequence analysis of soluble endosomal/lysosomal proteins. *D. discoideum* preprocathepsin D is composed of 383 amino acids. The short signal sequence (18 amino acids) is co-translationally removed upon translocation into the ER lumen, while the propeptide segment (from position 19 to 48) is removed later in endosomal compartments. Mature *D. discoideum* cathepsin D is a single-chain enzyme that shows a molecular weight of about 44 kDa. It mainly presents with only one *N*-linked oligosaccharide (on Asn118), although two other potential *N*-glycosylation sites (Asn238 and Asn310) are present in the protein (Journet et al. 1999).

### *4.10.3 Maturation of Cathepsin D*

The initial biogenetic events leading to the biosynthesis of *N*-glycosylated procathepsin D and its transport from the TGN to endosomes are substantially similar in all mammals, whereas the processing of procathepsin D into the mature enzyme(s) may present species-specific peculiarities in the generation of the single-chain and of the double-chain isoforms. Thus, in human and porcine cells, the single-chain polypeptide is rapidly processed and the double-chain enzyme is the most predominant molecular form of mature cathepsin D (Hasilik and Neufeld 1980; Erickson et al. 1981). The most abundant isoform of purified porcine cathepsin D is a double-chain protein formed by a 15-kDa light chain and a 31-kDa heavy chain, although a 43-kDa single-chain intermediate can also be isolated (Barth and Afting 1984). It was shown that porcine double-chain cathepsin D originated from the latter through the removal of five amino acids (Yonezawa et al. 1988). By contrast, in ovine, rat, hamster and mouse cells cathepsin D accumulates mainly as a single-chain isoform (Fujita et al. 1991; Isidoro et al. 1991, 1995; Tyynelä et al. 2000). In these mammalian species, the processing of the single-chain isoform into the double-chain isoform proceeds very slowly and, as a consequence, at steady state the latter represents less than 10–15 % of the whole cathepsin D isolated from acidic compartments. Of note, rodent cells possess the enzymatic machinery for the single- to double-chain processing of cathepsin D. In fact, human cathepsin D heterologously expressed in hamster cells is efficiently maturated into the double-chain isoform (Conner et al. 1989; Isidoro et al. 1991).

### 4.10.4 Mechanism and Physiological Significance of the Single- to Double-Chain Processing of Cathepsin D

Based on the observation that single-chain bovine cathepsin D converts into the double-chain form upon incubation at pH 3.5, it was suggested that the active intermediate molecule can process itself through an autocatalytic cleavage once it reaches the lysosome (Lah and Turk 1982). A more recent study proposes that human cathepsin D is processed into the double-chain form by cathepsins B and/or L (Laurent-Matha et al. 2006), which is consistent with earlier studies employing different cysteine cathepsin inhibitors (Gieselmann et al. 1985; Samarel et al. 1989). However, other cysteine cathepsins might also be involved in this process since cathepsin B/L double-knockout mice still contain normal levels of mature double-chain cathepsin D (Felbor et al. 2002; Stahl et al. 2007). Whether this processing occurs in endosomes before the single-chain intermediate is translocated into lysosomes or within the lysosome soon after the intermediate reaches this compartment is not yet fully clarified (Hasilik 1992). Recently, in an attempt to clarify the molecular mechanism and the nature of the acidic compartment(s) involved in this maturation step, a human cathepsin D mutant was studied in which the beta-hairpin loop excised during double-chain processing had been deleted (Follo et al. 2007). While wild-type human cathepsin D was efficiently maturated, thus establishing the lysosomal processing capacity of the recipient mouse cells, the cathepsin D mutant was not converted into the mature double-chain form although it reached the lysosome (Follo et al. 2007). These data argue against a quality control mechanism that would impair the transport of unprocessed intermediate cathepsin D from endosomes to lysosomes, and are rather compatible with the view that the last maturation step occurs within lysosomes.

What about the physiological relevance of the single- to double-chain processing? It can be hypothesized that these two cathepsin D variants have different substrate specificity. In general, single-chain cathepsin D seems more prone to act on small substrates and to be active at neutral or slightly acidic pH. Endosomal single-chain rabbit cathepsin D was shown to be able to process parathyroid hormone into bioactive peptides (Diment et al. 1989). Rat single-chain cathepsin D secreted at the basolateral site of mammary acini was shown to be able to mediate, at neutral pH, the cleavage of the hormone prolactin into bioactive peptides (Lkhider et al. 2004). During apoptosis associated with lysosomal membrane disruption, endosomal/lysosomal cathepsin D translocates into the cytosol and cleaves a so-far unknown substrate followed by activation of Bax (Bidère et al. 2003; Castino et al. 2007). *In vitro* cathepsin D activity is classically assayed using bovine haemoglobin, a rather complex substrate, at very acidic pH. Single- and double-chain cathepsin D isolated from bovine spleen were shown to possess different activity in such an assay, the double-chain form being more active towards haemoglobin at pH 3.5 (Lah et al. 1984). Similarly, Tanji et al. (1991) demonstrated that two different isoenzymes of cathepsin D purified from skeletal muscle of Japanese monkey differ in their specificity towards oxidized insulin β-chain.

Human double-chain cathepsin D also shows a higher affinity towards haemoglobin than its single-chain counterpart (Kawada et al. 1997). It is interesting to note that these authors found that single-chain cathepsin D was the most abundant form of the enzyme in epithelial cells of healthy skin, whereas the double-chain form was the major cathepsin D variant present in psoriatic skin biopsies. This observation would indicate that the extent of the cathepsin D maturation process likely depends on the tissue, the microenvironment and on particular pathophysiological conditions.

### *4.10.5 Structure and pH-Dependent Activation of Cathepsin D*

*In vivo*, procathepsin D is activated by acid-dependent proteolytic removal of the 44-residue propeptide culminating in generation of an active single-chain intermediate (see above). However, at least *in vitro*, procathepsin D can undergo another type of acid-dependent proteolytic activation that leads to 'pseudo'-cathepsin D which misses only the first 26 amino acids (Conner and Richo 1992). Pseudo-cathepsin D is enzymatically active (Gopalakrishnan et al. 2004) and retains the last 18 residues of the prosegment (amino acids 27–44). Interestingly, this residual fragment of the propeptide has been claimed to possess mitogenic properties (Vetvicka et al. 1998). Whether generation of pseudo-cathepsin D also occurs during pro-cathepsin D maturation *in vivo* is still debated (Richo and Conner 1994; Wittlin et al. 1999). It is also unclear whether this process is purely autocatalytic (Wittlin et al. 1999; Laurent-Matha et al. 2006). Although complete removal of the propeptide seems obligatory for cathepsin D to gain full enzymatic activity, *in vitro* studies indicated that at acid pH the propeptide does not occupy the active site, implying that procathepsin D could be active under these conditions (Wittlin et al. 1998). A better understanding of the pH-dependent mechanism of cathepsin D activation has been made possible by the elucidation of the three-dimensional structures of native and pepstatin-inhibited cathepsin D in combination with modelling studies (Baldwin et al. 1993; Metcalf and Fusek 1993; Lee et al. 1998; Goldfarb et al. 2005). By comparing the crystal structures obtained at pH 5.1 and 7.5, it was observed that an N-terminal segment (residues 3–7) relocated into the active site at neutral pH, thus explaining its inaccessibility for substrates or inhibitors at pH above 7.0 (Lee et al. 1998). A recent study proposes that the pH-dependent conformational change occurring in the molecule is initiated by charge repulsion between the two carboxylates of Glu180 and Asp187 (Goldfarb et al. 2005). Such a pH-dependent conformational switching may have physiological relevance for general and restricted cathepsin D-mediated proteolysis in terms of substrate specificity, compartmentalized activity, as well as for procathepsin D function as mitogenic ligand (Beyer and Dunn 1996; Berchem et al. 2002).

### 4.10.6 Cathepsin D in Biological Fluids and Pathological Conditions

Environmental stimuli (e.g. cytokines, growth factors, hormones, pH, hypoxia) or genetic factors (e.g. mutations in proteins involved in lysosomal protein traffic) may affect lysosomal targeting of cathepsin D or induce the exocytosis of the enzyme from endosomal-lysosomal compartments (Chiarpotto et al. 1999; Carini et al. 2004; Koo et al. 2008; Takenouchi et al. 2011). As a consequence, a substantial portion of procathepsin D and/or mature cathepsin D may be located extracellularly. This event is likely to occur for instance in cancer cells (Vashishta et al. 2005; Liaudet-Coopman et al. 2006), but also under certain physiological conditions such as allergen stimulation of mast cells (Dragonetti et al. 2000; Puri and Roche 2008), activation of dendritic cells following interaction with cytotoxic lympocytes (Gardella et al. 2001), bone remodelling (Czupalla et al. 2006), lactation (Lkhider et al. 2004; Castino et al. 2008) and luteal regression (Erdmann et al. 2007). Therefore, it is not surprising that cathepsin D, either as inactive precursor or as mature enzyme, can be found in biological fluids. Cathepsin D has been found in serum or plasma (Zühlsdorf et al. 1983; Naseem et al. 2005), milk (Větvicka et al. 1993; Larsen and Petersen 1995; Benes et al. 2002; Christensen et al. 2010), urine (Zühlsdorf et al. 1983) and even sweat (Baechle et al. 2006) or gastric juice (Ruan et al. 2011). The basal presence of cathepsin D in biological fluids probably merely reflects default lysosomal spillage into extracellular fluids and/or limited tissue damage. However, during abnormal 'leakage' from pathological tissues the plasma level of cathepsin D rises far above its physiological concentration and thus often represents a disease marker. Thus, for instance, abnormal levels of cathepsin D are found in the serum of cancer patients (Abbott et al. 2010; Szajda et al. 2008; Fukuda et al. 2005; Nicotra et al. 2010) and in the plasma of patients after myocardial infarction (Naseem et al. 2005). The physiological role of cathepsin D in milk is still obscure, but it is known that this protease is involved in coagulation and degradation of milk proteins (Larsen et al. 1996; Hurley et al. 2000). More recently, cathepsin D has been shown to process osteopontin in milk (Christensen et al. 2010). Cathepsin D found in urine presents with a glycosylation pattern different from that of its counterpart found in serum which suggests a local origin from renal tissue, and not from blood, as also supported by the elevated level of urinary cathepsin D in patients with nephritic syndrome (Zühlsdorf et al. 1983). The presence of active mature cathepsin D in eccrine sweat has been related to antimicrobial activity and immune defense on the skin surface (Baechle et al. 2006).

Abnormalities in the expression and secretion of cathepsin D have been reported in a variety of diseases, including psoriasis (Chen et al. 2000), atherosclerosis (Li and Yuan 2004), Alzheimer's disease (Cataldo et al. 1995; Zhou et al. 2006; Hamano et al. 2008), Parkinson's disease (Qiao et al. 2008; Cullen et al. 2009) and cancer (Nicotra et al. 2010). Cathepsin D appears implicated in all critical steps of cancer development and progression (Garcia et al. 1990; Isidoro et al. 1995; Liaudet

et al. 1995; Berchem et al. 2002; Benes et al. 2008; Ohri et al. 2008), including chemoresistance (Wu et al. 1998; Sagulenko et al. 2008; Castino et al. 2009) and formation of metastases (Liaudet et al. 1994; Glondu et al. 2002; Vashishta et al. 2007). It has been noticed that the role of cathepsin D in tumourigenesis is not only attributable to its proteolytic activity. In fact, an enzymatically inactive mutant of cathepsin D was shown to still possess mitogenic properties (Glondu et al. 2001). This mitogenic ability seems to reside on a fragment of the propeptide (amino acids 27–44) that interacts with a so far unknown membrane receptor (Fusek and Vetvicka 1994; Vetvicka et al. 1997, 2004).

## 4.11 Cathepsins in Lysosomal Storage Disorders

Lysosomal storage diseases comprise a heterogeneous group of about 50 inherited metabolic disorders. Many of these disease states can be attributed to the selective loss of a single hydrolase involved in the strictly ordered breakdown of glycoproteins, proteoglycans and sphingolipids. These deficiencies result in the gradual accumulation of the respective substrate within lysosomes, ultimately blocking the functions of these organelles (Neufeld 1991; Futerman and van Meer 2004). Surprisingly, only one lysosomal storage disease, pycnodysostosis, is due to a cathepsin deficiency. This disorder, manifested by osteosclerosis and short stature, is caused by mutations in the cathepsin K gene (Gelb et al. 1996). This is consistent with cathepsin K being the main collagenolytic proteinase produced by osteoclasts, as is evident from the analysis of cathepsin K-deficient mice (Saftig et al. 1998).

Another congenital disorder associated with a cathepsin deficiency is Papillon-Lefevbre syndrome, a hereditary disease characterised by hyperkeratosis and severe periodontitis. This disorder is due to genetic inactivation of cathepsin C (Toomes et al. 1999). However, as yet it is not clear which metabolic pathways are affected by the absence of the enzyme. In mice, disruption of the cathepsin C gene abolishes the cytolytic activity of T-lymphocytes, owing to interference with the activation of the destructive and pro-apoptotic serine proteinases granzymes A and B (Pham and Ley 1999) which requires cathepsin C-mediated removal of their N-terminal dipeptide proregions.

The neuronal ceroid lipofuscinoses constitute a group of neurodegenerative lysosomal storage diseases characterised by progressive psychomotor retardation, blindness and premature death. Interestingly, an inactivating mutation of cathepsin D accounts for a related disease in sheep (Tyynelä et al. 2000). Furthermore, disruption of the murine cathepsin F gene causes neuronal lipofuscinosis and neuropathological symptoms (Tang et al. 2006). Intriguingly, cathepsin F is the only cysteine cathepsin whose individual inactivation leads to such a lysosomal storage defect in mice. Animals deficient in both cathepsin B and cathepsin L die soon after birth due to massive brain atrophy and neuronal degeneration whereas

mice lacking either cathepsin B or cathepsin L are relatively healthy, indicating that these proteinases may functionally compensate for each other (Felbor et al. 2002).

## 4.12 Concluding Remarks

Cathepsins play a fundamental role in a wide range of important physiological processes. However, these enzymes also contribute to various devastating disease states. The latter is frequently associated with changes in cathepsin biosynthesis and intracellular transport. This chapter summarizes our current knowledge of the molecular mechanisms governing cathepsin trafficking and *in vivo* activation in the context of the pathophysiological significance of these potent proteases.

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