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Minireview

Regulation of human cathepsin B by alternative mRNA splicing: homeostasis, fatal errors and cell death

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Abstract

One of the control mechanisms of cathepsin B biosynthesis and trafficking operates through alternative splicing of pre-mRNA. An mRNA lacking exon 2 is more efficiently translated than that containing all exons, and may be responsible for elevated biosynthesis and enzyme routing to the extracellular space, with critical consequences for connective tissue integrity in pathologies such as cancer and arthritis. mRNA missing exons 2 and 3 encodes a truncated procathepsin B form that is targeted to mitochondria. This enzyme variant is catalytically inactive because it cannot properly fold. However, it provokes a cascade of events, which result first in morphological changes in intracellular organelles and the nucleus, finally leading to cell death.

Keywords: cysteine peptidases; mitochondria; overexpression; trafficking; unfolded protein; untranslated region.

Introduction

Cathepsin B, a cysteine peptidase of the papain family (EC 3.4.22.1, identifier C01.060 in the Merops database; Rawlings et al., 2004), has been typically classified as a lysosomal enzyme. However, it is also responsible for a variety of extracellular functions, is found at cell surfaces, and a truncated form is targeted to mitochondria. Some of these extralysosomal properties of cathepsin B are due to altered expression at the gene level, as well as to enzyme overproduction and/or atypical trafficking. Normal and abnormal expression can be regulated by transcriptional efficiency, by different transcription starting points (Gong et al., 1993; Berquin et al., 1995), by the use of alternative promoters (Yan and Sloane, 2003), and by variable pre-mRNA splicing (Gong et al., 1993; Berardi et al., 2001; Zwicky et al., 2002, 2003; Müntener et al., 2003, 2004).

During the splicing of eukaryotic precursor mRNAs, the introns are removed and the exons are joined together. Alternative mRNA splicing is a frequent event that occurs in a cell-specific or developmental-specific manner in approximately 60% of all human gene products (Modrek and Lee, 2002). It involves the combination of different splice junctions that give rise to functionally distinct proteins, allowing a gene to multiply its coding capacity and thereby to synthesize a number of structurally and functionally distinct protein forms far exceeding the number predicted by the genome. In this article we review our contribution to the study of alternative splicing of human cathepsin B pre-mRNA, with emphasis on the untranslated regions (UTR), and discuss the consequences of this event for protein synthesis, trafficking and function.

The cathepsin B gene, its transcripts and protein products

The human cathepsin B gene, located on the short arm of the 8p22 region of chromosome 8 (Fong et al., 1986; Wang et al., 1988), contains 13 exons (12 exons plus exons 2a,b) and has a coding portion of only approximately 1 kb, although its total length is at least 27 kb (Figure 1). Exons 2a,b (119 bp) have been discovered in cells from a human gastric adenocarcinoma (Berquin et al., 1995). Three cathepsin B transcripts, alternatively spliced in the 5′-region and relevant to this review, are shown in Figure 2. The standard 3′-UTR of cathepsin B mRNA contains the complete exon 11, giving rise to a 2.2-kb RNA, while a 4-kb variant contains 141 nucleotides of exon 11 and the complete exon 12 (Gong et al., 1993). Another 1.7-kb transcript results from splicing of the coding region to a more downstream portion of the 3′-UTR (Tam et al., 1994). The presence or absence of a 10-bp sequence in the 3′-UTR is crucial for stabilization of the transcripts through a stem loop (Tam et al., 1994).

As the regular start codon is located in exon 3, the full-length transcript, CB(full), and the transcript lacking exon 2, CB(-2), code for the same protein, namely preprocathepsin B. This consists of a 17-aa-long signal peptide, followed by an inhibitory propeptide of 62 aa, the single-chain enzyme (254 aa), and a C-terminal propeptide of 6 aa. The protein is directed to the endoplasmic reticulum by the signal peptide, which is then removed and the protein becomes glycosylated at two sites, one in the propeptide and one in the mature protein region. The mannose-6-phosphate recognition marker, added to the propeptide in the Golgi apparatus, conveys the enzyme to the endosomes, where the mature single-chain form of 27.8 kDa is generated by removal of the propeptide through limited proteolysis. Further processing occurs in the endosomal-lysosomal compartment, where the C-terminal hexapeptide and two internal amino acids are trimmed off, and the N-linked oligosaccharide of the heavy chain is degraded to the level of a single

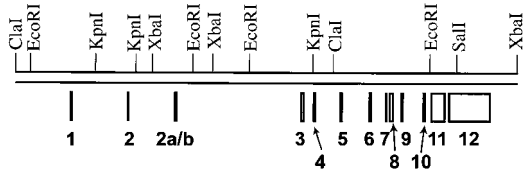

Figure 1 The human cathepsin B gene.
This scheme was generated using nucleotides 11760450–11791965 (in reverse order) of the chromosome 8 sequence of the *Homo sapiens* genome (build 30) as provided on www.ncbi.nlm.nih.gov and the pDraw32 software. The location of exon 2a/b was determined using the published sequence (Berquin et al., 1995). The boundaries of all other exons (shown by numbers) were determined using the published exon-intron junction sequences (Gong et al., 1993). Exon 1, 124 bp (5′-UTR); exon 2, 88 bp (5′-UTR); exon 2a/b, 119 bp (5′-UTR); exon 3, 151 bp (consisting of a 25-bp 5′-UTR and 126-bp coding region); exon 4, 86 bp; exon 5, 115 bp; exon 6, 119 bp; exon 7, 86 bp; exon 8, 144 bp; exon 9, 117 bp; exon 10, 129 bp; exon 11, 896 bp (consisting of a 98-bp coding region and 798-bp 3′-UTR); exon 12, ca. 2700 bp. The restriction sites are also shown.

N-acetylglucosamine residue. The mature lysosomal enzyme consists of a heavy and a light chain of 22.4 and 5.2 kDa, respectively, joined by a disulfide bridge (reviewed by Mort, 2004).

Translational efficiency

The question arises as to whether there is any special reason to be interested in the 5′-UTR variants CB(full) and CB(-2) if they encode the same protein. The answer is yes, especially as the 88-nt-long exon 2, which is part of an Alu element (Berquin et al., 1997), regulates the translational efficiency of the transcripts. To quantitatively measure this property, and in parallel the role of the 3′-UTR, we used constructs containing cathepsin B elements fused to the coding sequences of the green fluorescent protein or luciferase (Zwicky et al., 2003). We analyzed the expression of the alternative transcripts as functional proteins by quantitative confocal fluorescence microscopy in living cells and by luminescence analysis using four mammalian cell lines: T/C-28a2 immortalized chondrocytes, HeLa, CHO-K1 and COS-1 cells. As a general trend, constructs missing exon 2 were biosynthetically more efficient than the full-length counterpart in all cell types. Cloning the luciferase reporter upstream of the 3′-UTR, downstream of the 5′-UTR, or in between the 5′- and 3′-UTR enabled us to analyze the impact of the UTRs on cathepsin B expression. These UTRs downregulated luciferase biosynthesis moderately when present individually, with the 3′-UTR being more efficient than the 5′-UTR, but downregulated it even more when present simultaneously. Since these measurements only considered the effect of parts of the 5′-UTR of cathepsin B mRNA in expressing reporter proteins, and might thus be considered somewhat artificial, we addressed the question of the translational efficiency of the CB(full) and CB(-2) transcripts using antisense methods. We designed an antisense nucleotide consisting of 24 bases specifically designed to hybridize with CB(-2) by binding 12 bases each on exon 1 and exon 3, and considered the appropriate controls (Zwicky et al., 2002). Application of this antisense oligonucleotide to a human immortalized chondrocyte cell line, T/C-28a2, the mRNA of which contained only 6% of the CB(-2) splice variant, showed that this transcript was responsible for 30–50% of the total biosynthetic activity. Considering these results and the outcome for the reporter proteins described above, we conclude that the translational efficiency of CB(-2) can range between two- and 15-fold higher than that of CB(full), depending on the conditions and the particular cell.

The transcript lacking both exons 2 and 3, CB(-2,3), can be translated because of a second in-frame start codon present in exon 4 (Figure 2). However, this transcript leads to a truncated form of procathepsin B that lacks the signal peptide (necessary for import into the endoplasmic reticulum) and 34 residues of the inhibitory propeptide. Using fusion constructs containing green fluorescent protein and luciferase to monitor protein biosynthesis, the translational efficiency of CB(-2,3) was shown to be maximally 4.5-fold higher than that of CB(full), depending on the cells (Zwicky et al., 2003).

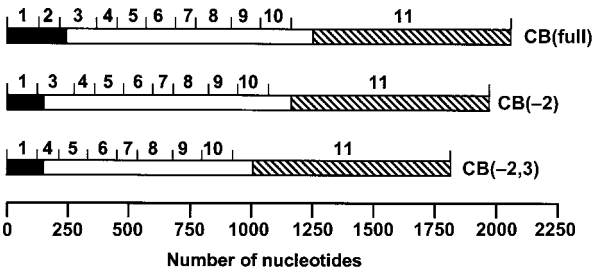

Figure 2 Alternative cathepsin B messages in the 5′ region and their protein products.
Exons are drawn to scale. Here only the standard 3′-UTR is shown, which contains the complete exon 11. An alternative 3′-UTR contains 141 nucleotides of exon 11 and the complete exon 12. Black, 5′-UTR; hatched, 3′-UTR; white, coding region.

Occurrence of alternative cathepsin B transcripts in cells and tissues

There are only a few reports on the occurrence of alternatively spliced cathepsin B transcripts in human tissues (Gong et al., 1993; Berquin et al., 1995; Lemaire et al., 1997; Hizel et al., 1998; Berardi et al., 2001). The CB(full) and CB(-2) transcripts are found in variable proportions in normal and pathological tissues, with a trend to overexpression of CB(-2) in tumors (Gong et al., 1993; Hizel et al., 1998). The CB(-2,3) variant, originally presumed to be a rare tumor species (Gong et al., 1993), is also found in normal and rheumatoid synovial tissue (Lemaire et al., 1997), as well as in normal and osteoarthritic cartilage (Berardi et al., 2001). The relative proportions of CB(full), CB(-2) and CB(-2,3) in normal and osteoarthritic cartilages vary from specimen to specimen, but there is a trend to an increase in the amount of CB(-2) and CB (-2,3) in pathological tissues (Berardi et al., 2001) compared to normal tissues. However, such statistical considerations, which refer to bulk quantitative analyses in the cartilage of a whole joint, do not make much sense. In fact, *in situ* investigation by RT-PCR, aimed at mapping the occurrence of the three transcripts through the depth

of articular cartilage from the surface down the subchondral bone, revealed remarkable differences in their distribution. While all transcripts were preferentially found in the vicinity of the articular surface in normal cartilage, they were distributed through the depth of the tissue in osteoarthritic cartilage, with evident accumulation at 'more involved' sites (Berardi et al., 2001). This finding agrees with independent localization results for cathepsin B activity by enzyme histochemistry (Baici et al., 1995a,b).

Besides variations in the relative proportions of the 5′-UTR alternatively spliced transcripts, a trend towards general enhancement of cathepsin B mRNA levels is observed in pathological tissues compared to normal counterparts (Berardi et al., 2001; Yan and Sloane, 2003). Under homeostatic conditions, the total level of cathepsin B mRNA results in enzyme expression and trafficking, which is efficiently controlled. Considering an increase in both the total mRNA level and in the proportion of the splice variant CB(-2), a quiet housekeeping function can be ascribed to the CB(full) message, while CB(-2) appears to be responsible for uncontrolled enzyme overproduction and misrouted trafficking.

Trafficking

Transient transfection experiments with cathepsin B constructs fused to the green fluorescent protein were performed in human immortalized chondrocytes and HeLa cells. Co-localization with the *trans*-Golgi network and the acidic compartment was monitored using specific markers. As expected, the product of CB(full) and CB(-2) appeared in the *trans*-Golgi network and from there it was delivered to the endosomal-lysosomal compartment as its final location (Müntener et al., 2003). Inclusion or omission of the 6-aa-long C-terminal propeptide had no influence on cathepsin B trafficking. Mutation of the glycosylation site Asn 38 to Gln prevented the necessary glycosylation for targeting the nascent protein to the lumen of the endoplasmic reticulum, and the product of this construct was first detected in the rough endoplasmic reticulum before being directly delivered to the secretory pathway. However, in contrast to breast carcinoma cells (Moin et al., 2000), we could not confirm the existence of an alternative, mannose-6-phosphate receptor-independent delivery path to the acidic compartment in chondrocytes and HeLa cells. There is indeed a difference between our approach and that of Moin et al. in including (Müntener et al., 2003) or not including (Moin et al., 2000) elements of the 5′-UTR in the constructs with green fluorescent protein.

After transient transfection of cells with the constructs CB(full) and CB(-2) as just described, regular targeting of procathepsin B to endosomes and lysosomes was evident, and we also observed its extracellular release. Secretion was enhanced in the case of overexpression and was particularly evident with constructs lacking exon 2 from the 5′-UTR. This property correlates with the known secretion of procathepsin B in cases of overexpression, as documented, for instance, in tumors (Frosch et al., 1999) and osteoarthritis (Berardi et al., 2001). In addition to observing procathepsin B secretion from osteoarthritic and dedifferentiated chondrocytes in culture, we could extract intact glycosylated procathepsin B from human osteoarthritic cartilage (Berardi et al., 2001). We have demonstrated that procathepsin B accumulates in pathological cartilages by binding to the extracellular matrix, and that proenzyme contact with sulfated glycosaminoglycans promotes its conversion to the active form, which can be detected using a specific enzyme histochemical assay (Baici et al., 1995b).

Without knowing further details, it could be supposed that truncated procathepsin B, the product of the splice variant CB(-2,3), is rapidly eliminated after biosynthesis because it cannot be conveyed to the endoplasmic reticulum, modified in the Golgi apparatus or sent to the lysosomal or to the secretory pathway. Early *in vitro* results that reported the artificial expression of truncated procathepsin B in COS cells and folding into an active enzyme (Mehtani et al., 1998) could not be confirmed by our recent studies (Müntener et al., 2005). After observing a cytoplasmic and structured localization of green fluorescent protein following transfection of chondrocytes and HeLa cells with a cathepsin B construct missing exons 2 and 3 fused to green fluorescent protein (Müntener et al., 2003), a detailed study allowed us to track the final destination of truncated procathepsin B to a previously unsuspected location, the mitochondrion (Müntener et al., 2004). Co-localization of the truncated procathepsin B-green fluorescent chimeras with mitochondria was demonstrated by specifically labeling these organelles with MitoTracker red, which fluoresces red in metabolically active mitochondria (Figure 3, left).

How truncated procathepsin B enters mitochondria and the consequences

Close examination of the N-terminal sequence of truncated procathepsin B, which starts with Met 52, reveals

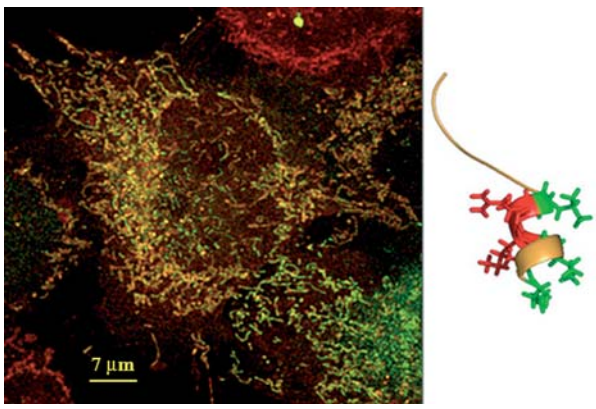

Figure 3 Human truncated procathepsin B in mitochondria. (Left) A cathepsin B-green fluorescent protein chimera containing the first 65 amino acids of truncated procathepsin B starting with position 52 (aa numbering of preprocathepsin B) was induced in HeLa cells. Confocal fluorescence microscopy shows in red metabolically active mitochondria stained with MitoTracker red and in green the cathepsin B chimera. The yellow color indicates co-localization of the red and green signals. (Right) The N-terminal segment of human truncated procathepsin B showing a typical mitochondrial import motif, which consists of an amphipathic α-helix with a positively charged patch (red) opposite to a hydrophobic patch (green).

the presence of a typical leader sequence for the import of proteins into the mitochondrial matrix (Chacinska et al., 2002). This consists of a small patch of hydrophobic amino acids, opposite to a patch of positively charged amino acids in an amphipathic α-helix, as shown in Figure 3 (right). This Figure was drawn from the published structure of procathepsin B (Turk et al., 1996) by deleting amino acids up to position 51 and showing 15 residues, starting at position 52. Only the side chains of the residues that make up the helix are explicitly represented. The propensity of this segment of the molecule to form an amphipathic helix in truncated procathepsin B was confirmed by computation using the program PSIPRED (McGuffin et al., 2000). Besides the co-localization experiments described above and illustrated in Figure 3, we directly verified the presence of truncated procathepsin B inside mitochondria after isolation of subcellular particles of transfected HeLa cells by differential centrifugation, followed by Western blot analysis. Further evidence that truncated procathepsin B is delivered to the mitochondrial matrix comes from the presence, in Western blots, of two bands shortened by 2.5- and 4.4-kDa peptides with respect to the molecular mass of the chimera (Müntener et al., 2004). It is strongly suspected that such shortening is due to the action of mitochondrial processing peptidase, the enzyme responsible for cleaving off the import presequences of proteins targeted to the mitochondrial matrix. This enzyme preferentially catalyzes cleavage at positions having an arginine as the penultimate residue (-2) with an aromatic side-chain at the next position (+1) (Chacinska et al., 2002; Braun and Schmitz, 2004). There are two such potential cleavage sites in the N-terminal segment of truncated procathepsin B, namely between aa 73–74 and 89–90 (preprocathepsin B numbering).

Inside the mitochondrion, instead of behaving as an innocent bystander, truncated procathepsin B has deleterious consequences for cell integrity. Morphological alterations are observed in the *trans*-Golgi network, in the acidic compartment, in mitochondria and, most prominently, in the nucleus, which undergoes drastic deformation followed by cell death (Müntener et al., 2003, 2004; Zwicky et al., 2003). Although such properties might be attributed to proteolytic events on the part of truncated procathepsin B, we demonstrated that this enzyme form is catalytically inactive (Müntener et al., 2005). The propeptide of cathepsin B exerts a double function: as a chaperone it assists correct folding of the nascent protein, and as an inhibitor it prevents substrate binding by running its chain over the active center in a direction opposite to that of a regular peptidic substrate. We tested the capability of procathepsin B to fold and to become enzymatically active by expressing protein variants lacking up to 22 amino acids at the N-terminus in baculovirus-infected insect cells. The Asp^{11}–Arg^{20} α-helix (proenzyme numbering) is necessary for efficient inhibitory activity of the propeptide. Trimming away this element or additional amino acids abolishes inhibition. Proenzyme variants, from which the N-terminal part including the Trp^{24}–Ala^{26} β-sheet is missing, or which contain an amino acid mutation directly preceding this β-sheet, are unable to fold (Müntener et al., 2005). Therefore, procathepsin B forms lacking more than 22 amino acids at the N-terminus would potentially be enzymatically active, but they are destined to remain inactive because they cannot fold correctly. Truncated procathepsin B, lacking 34 amino acids of the propeptide, has thus no chance of folding and of becoming an active enzyme.

A likely hypothesis to explain the deleterious effects of truncated procathepsin B on cell integrity is the accumulation of unfolded protein within the mitochondrial matrix. Figure 3 clearly reveals the structure of mitochondria, organelles existing in two interconverting forms: as small isolated, rounded particles (the typical textbook image), and as extended filaments. These extended mitochondria represent electrically connected units, which facilitate energy delivery from the cell periphery to the cell core. They function as intracellular power-transmitting cables organized as a mitochondrial reticulum (Skulachev, 2001). Careful analysis of confocal images such as that shown in Figure 3 reveals changes in the structure of the mitochondrial reticulum, and the effects produced by the accumulation of unfolded truncated procathepsin B can be visualized after dissecting the co-localization features into the component colors (Müntener et al., 2004). The physical integrity of mitochondria as a system of energy-delivering cables is essential for their performance. We believe that accumulation of unfolded truncated procathepsin B induces the cell to physically fragment its mitochondrial reticulum as a protective mechanism to prevent short circuit in the whole electrical network. However, this reaction ultimately results in power failure and termination of energy delivery. The subsequent morphological changes reveal features that resemble apoptosis rather than necrosis. However, we cannot precisely assign them to a given cell-death mechanism. They can possibly be placed somewhere in one of the categories of cell death described in a variety of physiological and pathological situations, such as autophagy (Bröker et al., 2005), oncosis (Otsuki et al., 2003) or paraptosis (Sperandio et al., 2000). Unfortunately, a detailed study of the mechanism of cell death induced by truncated cathepsin B is currently impossible, as this represents a process that occurs slowly in an asynchronous way in only a few cells at a time. The low frequency of this process might explain the slow depletion of cells in tissues undergoing degeneration over many years, such as osteoarthritic cartilage. In this pathological situation, characterized by overexpression of cathepsin B at both the mRNA and protein level and with a clear presence of the CB(-2,3) mRNA splice variant (Berardi et al., 2001), cell death is a rare phenomenon, but this would be sufficient to deplete articular cartilage of its cells in one or two decades. Extending this concept, we can postulate a function of truncated procathepsin B as a physiologically relevant mechanism of cell death in tissues characterized by slow turnover and populated by long-lived cells.

Acknowledgments

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