Folding Competence of N-terminally Truncated Forms of Human Procathepsin B

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Besides acting as an inhibitor, the propeptide of human cathepsin B exerts an important auxiliary function as a chaperone in promoting correct protein folding. To explore the ability of N-terminally truncated forms of procathepsin B to fold into enzymatically active proteins, we produced procathepsin B variants progressively lacking N-terminal structural elements in baculovirus-infected insect cells. N-terminal truncation of the propeptide by up to 22 amino acids did not impair the production of activable procathepsin B. Secreted forms lacking the first 20, 21, or 22 amino acids spontaneously generated mature cathepsin B through autocatalytic processing, demonstrating that the first α-helix (Asp11–Arg20) is necessary for efficient inhibition of the enzyme by its propeptide. In contrast, proenzymes lacking the N-terminal part including the first β-sheet (Trp24–Ala26) of the propeptide or containing an amino acid mutation directly preceding this β-sheet were no longer properly folded. This shows that interactions between Trp24 of the propeptide and Tyr183, Tyr188, and Phe180 of the mature enzyme are important for stabilization and essential for procathepsin B folding. Thus, proenzyme forms missing more than the N-terminal 22 amino acids of the propeptide (notably truncated cathepsin B produced by the mRNA splice variant lacking exons 2 and 3, resulting in a propeptide shortened by 34 amino acids) are devoid of proteolytic activity because they cannot fold correctly. Thus, any pathophysiological involvement of truncated cathepsin B must be ascribed to properties other than proteolysis.

The cysteine peptidase family C1 (the papain family, mops.sanger.ac.uk) contains two groups of enzymes within subfamily C1A characterized by the length of their propeptides: cathepsin B-like and cathepsin L-like enzymes. They show very little sequence homology in their proregions, and in contrast to cathepsin L-like proenzymes, which have a propeptide of about 100 amino acids in length, the propeptide region of the cathepsin B-like proenzymes is about 60 amino acids long (1). Studies on cathepsins B, L, and S demonstrated that the propeptides or parts of them are effective inhibitors of their cognate enzymes (2–5). The three-dimensional structure of human procathepsin B shows that the propeptide (Arg1p-Lys62p, where p stands for propeptide) is jacketed around the catalytic domain of the enzyme in a C-shaped fold, positioned in the direction opposite to bound substrates, thereby shielding the active site (6). Two regions of rat procathepsin B were found to be particularly important for inhibition, the NTTWQ sequence spanning residues 21p–25p, and the CGTVL sequence (amino acids 42p–46p) (2).

Besides their function as intramolecular inhibitors, propeptides of several peptidases have been shown to assist in folding. Early in vitro refolding experiments showed that deletions in the propeptide of human cathepsin L resulted in the loss of enzymatic activity due to the lack of refolding capacity of the mature enzyme (7). In addition, the deletion of most of the prosegment of Fasciola hepatica cathepsin L1 resulted in a drastic decrease of secreted active enzyme (8), and deletion/mutagenesis studies of mouse cathepsin L and human cathepsin S also confirmed the folding competence of the propeptide of cathepsin L-like peptidases (9, 10). Conversely, the propeptide of cathepsin X, which consists of only 38 amino acids, was assumed to be unable to induce protein folding but is thought to stabilize the conformation of the N-terminal domain of the enzyme by forming a disulfide bridge between the proregion and the active site cysteine (11). The chaperone function of the cathepsin B propeptide was not thoroughly investigated so far. Truncated procathepsin B, referred to as “truncated cathepsin B” according to the literature, which is encoded by an alternatively spliced mRNA and lacks the signal peptide and 34 amino acids of the propeptide, was originally described as a catalytically active enzyme in vitro (12).

In this study, we investigate the function of the human cathepsin B propeptide as a chaperone, putting particular emphasis on the folding competence of truncated cathepsin B. To identify the structural elements necessary for the chaperone activity, we produced N-terminally shortened forms of human procathepsin B progressively lacking structural propeptide elements or carrying mutations that abolish Nglycosylation in insect cells using the baculovirus expression system. We discuss the importance of the interaction between Trp24p and the mature enzyme for proper protein folding assistance and provide evidence for the crucial function of the first N-terminal α-helix for intramolecular inhibition. We also show that truncated cathepsin B is not able to correctly fold into an enzymatically active conformation.

EXPERIMENTAL PROCEDURES

Plasmids—The human cathepsin B mRNA, to which all sequences in this study refer, is the sequence of 1996 nucleotides deposited under accession number L16510 at EMBL/GenBankTM/DDBJ. The structure of the clones used in this study, the corresponding protein products, and their putative subcellular localization are shown in Table I and in Fig.
were harvested from the suspension by centrifugation at 400 g. Virus was used to infect Sf21 cells grown in the same medium. Cells were incubated for another 3 h at 37 °C. Thereafter, supernatants were sterile-filtered (0.22 μm) prior to use to remove residual cellular debris. As negative controls, uninfected Sf21 cells or cell culture media were used. Recombinant wild type procathepsin B was purified from the respective culture supernatant essentially as described previously for N-acetylgalactosaminyl-transerase I (15), with the purified proenzyme being stored in phosphate-buffered saline containing 0.02% sodium azide. For pepsin activation of latent cathepsin B, 1 μl of 30 U/ml of protease was added to 50 μl of the supernatant in a final volume of 50 μl. The activity of the heterologous enzyme Cathepsin B Activity Assay—The activity of the heterologous enzyme was measured in both cell lysates and culture media. Cell lysates were prepared by resuspension in 50 mM sodium phosphate buffer containing 0.15 M NaCl and 0.5% SDS, 50 mM sodium citrate containing 0.02% sodium azide. For pepsin activation of latent cathepsin B, 1 μl of 30 U/ml of protease was added to 50 μl of the supernatant in a final volume of 50 μl. The reaction was stopped by precipitating 5 μl of lysate with 100 μl of phosphate-buffered saline, added to the cell pellet, and centrifuged again. The supernatant was discarded, and the pellet was washed twice in phosphate-buffered saline, centrifuged as in the preceding step, and stored at −20 °C.

Western Blot Analysis—Cell lysates and supernatants were mixed with reducing sample buffer, and SDS-PAGE was carried out on a 12% gel. Bands were detected via Western blot analysis using anti-cathepsin B (16), and the secondary antibody was peroxidase-conjugated rabbit anti-sheep IgG (Calbiochem-Novabiochem). Bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantitated using the磷脂ase A2 assay kit (G-3791, Sigma). The phospholipase assay is based on the number of units hydrolyzing dioleoylphosphatidylcholine (DOPC) in 2 min to form diacylglycerol. The phospholipase assay was carried out according to the manufacturer's instructions. For the determination of the phospholipase activity, 200 μl of sample was added to 200 μl of 16 mM dioleoylphosphatidylcholine (DOPC) in 50 mM sodium phosphate buffer pH 7.5 and 0.02% sodium azide at 37 °C. The reaction was stopped by adding 0.1 M HCl to 50 μl of the supernatants to lower the pH to ~3.0. Cell lysates were treated by incubating 5 μl of lysate with 100 μl of pepsin solution (0.125 μg/μl in 0.1 M glycine/HCl buffer, pH 3.2) for 12 min at 37 °C. The substrate solution consisting of 0.1 mM N-benzoyl-oxycarbonyl-Val-Arg-Arg-(4-methyl)-coumarylamide (Bachem, Bubendorf, Switzerland) in 50 mM sodium phosphate buffer containing 2 mM EDTA and 2 mM diethiothreitol, pH 6.0, was added to either pepsin-treated or untreated cell suspension or supernatant. To verify the specificity of the reaction, the cathepsin B-specific inactivator E-64 (Bachem) was added at a final concentration of 10 μM. Incubation took place under shaking in the dark at room temperature. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured at various incubation times in a Bio Assay Reader HTS 7000 Plus (PerkinElmer Life Sciences) reader at 430 nm after excitation at 380 nm.

1 The abbreviations used are: mCB, mature cathepsin B; proCB, procathepsin B; tCB, truncated cathepsin B; ER, endoplasmic reticulum; GFP, green fluorescent protein; PNGase, peptide-N-glycosidase F; Endo-H, endoglycosidase H; WT, wild type.
RESULTS
Baculovirus-mediated Expression of Truncated Procathepsin B Forms—The baculovirus expression vector system was used to obtain secretion of newly synthesized recombinant proteins into the culture medium. The secretion of these foreign proteins by insect cells is achieved by replacing the foreign signal peptide sequences with the signal peptide sequence derived from the secretory insect protein melittin (14, 15). As in mammalian cells, only properly folded proteins are able to pass through the secretory pathway of insect cells and undergo terminal N-glycan processing (17). In contrast, misfolded or unassembled proteins targeted to the secretory pathway are retained within the endoplasmic reticulum (ER). Therefore, cell extracts and supernatants of baculovirus-infected cells expressing different N-terminally truncated versions of human cathepsin B were analyzed by Western blot to assess their secretion status and thus the folding competence of the respective recombinant protein. Additionally, to analyze cytosolic expression and the influence of GFP attached to tCB on proper folding, proCB, tCB, tCBGFP, and mCB were expressed in the cytosol of insect cells by omitting the signal peptide. A short digest with pepsin was used to activate the latent proenzymes. In this assay, only correctly folded procathepsin B is converted into an enzymatically active form, whereas destabilized variants are completely digested with pepsin (19).

As shown in Fig. 2A, ProCB-2, -5, -9, -20AA, and wild type ProCB targeted to the secretory pathway are indeed secreted into the medium, as revealed in each lane by a band around 40 kDa (lanes 1–4 and 8), which was ascribed to procathepsin B. In lanes 4 and 8, another band at about 33 kDa was detectable, representing the mature single chain form of cathepsin B,
which arose from autocatalytic activation. In lanes 5 and 6, containing ProCB-23 and -26AA, no bands could be detected even if four times more culture medium was loaded onto the gel (not shown).

Fig. 3 shows the culture media of SF 21 cells expressing the secretory proteins ProCB-20AA, ProCB-20N21Q, ProCB-20T23A, ProCB-21AA, or ProCB-22AA and a variant of tCB containing a signal peptide. In each lane of Fig. 3A, except for those for ProCB-20T23A, tCB, and the uninfected cells, a band around 40 kDa was visible and represented cathepsin B. The secreted proteins were produced to different extents and showed partial activation as seen by the band around 33 kDa corresponding to mature single chain cathepsin B. To exclude that this mature form of cathepsin B arose through autocatalytic processing during the warm-up phase of heat inactivation, the experiments were repeated by adding the cysteine peptidase inactivator E-64 at a final concentration of 0.14 mM to the samples before heating and analyzing them again by Western blotting (not shown). The culture medium of the SF 21 cells expressing ProCB-20, -20N21Q, -21, and -22AA showed exactly the same pattern of bands as before. Consequently, these proenzymes are indeed prone to spontaneous autoactivation by virtue of their shortened proregion.

In the blots in Figs. 2B and 3B, showing the pepsin-treated samples, only bands around 33 kDa are visible, demonstrating that the propeptides were completely removed by pepsin. This also demonstrates that the secreted proteins were correctly folded because pepsin activates only properly folded procathepsin B, whereas it degrades destabilized or misfolded forms. In Fig. 2B, lanes 5 and 6, showing secretory forms of ProCB-23 and -26AA, respectively, no bands could be detected on the blot since these procathepsin B forms were quantitatively retained within the insect cells (see also Fig. 7). However, ProCB-2, -5, -9, and -20 AA were properly digested into the single chain form of cathepsin B.

Fig. 3B shows that the secretory proteins ProCB-20, -20N21Q, -21, and -22 AA (lanes 1, 2, 4, and 5) can be activated by pepsin. This points out that they are properly folded and therefore secreted by the cells, contrary to the similarly produced variants ProCB-20T23A (lane 3) and tCB (lane 7), which were not secreted at all. The faint bands at around 45 kDa visible in blot B in every lane represent pepsin. Surprisingly, the proregion lacking 22 amino acids was still able to act as a chaperone, whereas the proregion in which the amino acid at position 23 was mutated lost its chaperone function. As shown in Fig. 3, lane 7, tCB targeted to the secretory pathway was not secreted into the supernatant of the cells, not even if it was fused to GFP (data not shown). Pepsin treatment of the cell lysate containing secretory tCBGFP resulted in the complete digestion and disappearance of the band around 60 kDa (Fig. 4, compare lanes 1a and 1b). In addition, the cells did not display any GFP fluorescence. This finding indicates that secretory tCBGFP was misfolded.

**Cytosolic Expression—**ProCB, mCB, tCB, and tCBGFP were also expressed in the cytosol of insect cells by omitting the signal peptide. All cytosolically produced proteins were present in large amounts in the cells (Fig. 4, lanes 2a–5a). Cells expressing tCBGFP in the cytosol fluoresced (material applied to lane 5a), showing that GFP was correctly folded in the chimera. However, following pepsin digestion, no bands were left (Fig. 4, lane 5b), showing that besides GFP, which denatures under the acidic conditions of pepsin activation, the remaining truncated cathepsin B protein was also not correctly folded. In addition, ProCB, mCB, and tCB were all unable to correctly fold in the cytosol as shown in Fig. 4, lanes 2–4.

**Cathepsin B Activity Measurement—**Samples of the pepsin-treated and untreated proenzymes used in Figs. 2 and 3 were assayed to measure the cathepsin B activity of the secretory
and of the cytosolically expressed enzyme forms. This handy method allowed the qualitative assessment of the chaperone and inhibitory function of the proregion. To confirm the cathepsin B specificity of the reaction, measurements were also carried out in the presence of the cathepsin B-specific inactivator CA-074. The secreted enzyme forms ProCB-2, -5, -9, and -20 AA were clearly active, contrary to ProCB-23AA and ProCB-26AA, which did not seem to be correctly folded and secreted when targeted to the secretory pathway, as shown by a cathepsin B activity indistinguishable from background values (Fig. 5). As already seen in Figs. 2 and 3, ProCB-20AA, ProCB-20N21Q, ProCB-21AA, and ProCB-22AA were partially autoactivated and therefore showed activities even without pepsin activation. In Fig. 5, the autoactivation of ProCB-20N21Q, ProCB-21AA, and ProCB-22AA is hard to perceive as compared with ProCB-20AA because the former three proteins were secreted at a much lower level. WT-ProCBGFP targeted to the secretory pathway was indeed secreted by the cells and was active after pepsin treatment (Fig. 6). In contrast, secretory tCBGFP was quantitatively retained within the cells in an inactive state as shown in Fig. 4, lanes 1a and 1b. Similarly, all cathepsin B forms produced in the cytosol of the insect cells were devoid of enzymatic activity (Fig. 6).

Cleavage of N-Glycans from Procathepsin B—This deglycosylation experiment was aimed at determining the type of N-glycosylation, thereby validating the ability of the various proenzyme forms to fold properly. Preprocathepsin B translocates cotranslationally into the rough ER, where the signal peptide is removed and N-linked high mannose oligosaccharides are attached to the glycosylation sites. Only the properly folded proenzyme can be transported from the ER to the Golgi apparatus, where its N-glycans are further processed to complex N-linked oligosaccharides. If a protein is retained in the ER, it contains only high mannose N-glycans and thus remains Endo-H-sensitive, whereas secreted glycoproteins usually are resistant to cleavage by this enzyme. Although Endo-H only cleaves high mannose N-glycans and thus remains Endo-H-sensitive, whereas secreted glycoproteins usually are resistant to cleavage by this enzyme. Although Endo-H only cleaves high mannose N-linked oligosaccharides, N-glycosidase F is also active on hybrid and complex-type N-glycans. Secreted ProCB-9AA was activable by pepsin. Therefore, it should be...
Endo-H-insensitive but PNGase-sensitive. Indeed, a band around 40 kDa is present in Fig. 7, lanes 1a and 1c, showing the untreated protein and the Endo-H-treated protein displaying the same electrophoretic mobility. The band in lane 1b has a lower apparent molecular mass and represents deglycosylated ProCB-9AA obtained by treatment with PNGase F. The same pattern was found in lanes 7a–7c, containing a lysate of cells expressing secretory ProCB-9AA. These results show that secreted ProCB-9AA, as well as the proenzyme remaining in the cell, were not Endo-H-sensitive, thus confirming their correct folding. Secreted ProCB-20AA, ProCB-21AA, and ProCB-22AA were similarly insensitive to Endo-H treatment, but the intracellular proenzymes were mostly Endo-H-sensitive (Fig. 7, lanes 3a–3c and lanes 8a–8c). Consequently, the proenzymes lacking 20, 21, and 22 amino acids apparently have more difficulties to fold properly. The glycosylation site mutant ProCB-20N21Q showed the same behavior, whereas the ProCB-20T23A mutant was unable to fold correctly and therefore could not be detected in the supernatant. This can be explained with structural changes in the propeptide region due to alanine instead of threonine at position 23. Supernatants of cells expressing secretory forms of ProCB-23AA and -26AA were concentrated 25 times before being applied to the gel, but there were still no bands visible. ProCB-23AA and -26AA targeted to the secretory pathway were visible as strong bands in the cell lysates, clearly showing Endo-H sensitivity (Fig. 7, lanes 11 and 12). Secretory tCB, which is not secreted by the cells, suggesting that it is misfolded, revealed also Endo-H sensitivity (Fig. 7, lanes 13a–13c).

**DISCUSSION**

The baculovirus protein expression system in insect cells, which share many similarities with mammalian cells, represents a very useful tool for investigating biosynthesis, posttranslational processing, and folding of mammalian proteins. In addition, the level of endogenous cathepsin B in these cells is extremely low. In the baculovirus system, only correctly folded proteins follow the secretory pathway and are released into the cell supernatant. On the contrary, unfolded or misfolded proteins cannot be secreted because they are retained in the ER. Since ER retention results in altered N-glycosylation, the folding competence of recombinant proteins, such as cathepsin B, can be monitored by checking their glycosylation state.

We investigated the properties of the human cathepsin B proregion as a chaperone and as an intramolecular inhibitor using a systematic deletion approach of selected amino acid sequences starting at the N terminus of the molecule. According to the known secondary structure of the cathepsin B propeptide, variants missing 2, 5, 9, 20, 21, 22, 23, 26, and 34 (tCB) amino acids and two mutant forms aimed at preventing N-glycosylation, namely asparagine 21 to glutamine and threonine 23 to alanine, were expressed in insect cells using the baculovirus system. These particular mutated constructs were aimed at showing whether the amino acid sequence or the glycosylation is responsible for correct folding. Analysis of cell culture supernatants showed that all variants except for those lacking more than 22 amino acids or containing a mutation at this position could be correctly folded and secreted. Thus, the region around amino acid 23 is critical for cathepsin B folding. Interestingly, secreted forms lacking 20, 21, or 22 amino acids spontaneously generated mature cathepsin B through autocatalytic processing. The mutant form ProCB-20N21Q, lacking the N-glycosylation site, was also correctly folded, which shows that proper protein folding is not dependent on glycosylation of the propeptide.
Our results highlight the importance of specific elements of the propeptide of human cathepsin B that are responsible for its chaperone and intramolecular inhibitory function. In contrast to in vitro unfolding/refolding studies derived from the prorogation of human procathepsin S and human procathepsin L (7, 20), the insect cell system allows the assessment of protein folding under physiological conditions. Since folding was impaired in the case of the propeptide shortened by 23 amino acids and in the case of the propeptide mutated at position 23, we suggest that hydrophobic interactions between tryptophan 24p and tyrosine 183, as well as tyrosine 188 and phenylalanine 180, are necessary for folding and for anchoring the propeptide at the enzyme surface as suggested by Turk et al. (21). In addition, tryptophan 24p was shown to form a hydrogen bond with the carbonyl group of glutamine 189 (21). We consider that the sequence N-terminal to the tryptophan anchor could be responsible for fastening the proregion at the surface of the cathepsin B molecule, whereas a single amino acid can no longer sustain this function as evident from the propeptide variant shortened by 23 amino acids. A mutation at position 23, e.g. Thr to Ala as in this study, possibly leads to major structural differences with respect to the wild type protein, which hinders the interaction of the proregion with the enzyme surface. A similar stabilization of the enzyme structure through hydrophobic interactions was also demonstrated for cathepsin S (22).

Removal of 20 amino acids resulted in a propeptide with reduced inhibitory competence and thus favored autocatalytic processing to the mature form of cathepsin B. This suggests a key role of the α-helix (D11p-R20p) for proper inhibitory activity of the propeptide. If this helix is missing, destabilization of the propeptide is likely to occur, accompanied by insufficient protection of the active site and ending up with autocatalytic processing.

Truncated cathepsin B, the product of the mRNA splice variant missing exons 2 and 3 and lacking the first 34 N-terminal amino acids of the propeptide (23), was claimed to fold into an enzymatically active species in vitro (12). Our present results do not support this point of view since deletion of more than 22 amino acids of the propeptide invariably resulted in inactive cathepsin B forms, even when forced into the cytosolic expression pathway. Although being unable to be secreted and to assume an active conformation in the reducing milieu of the cytosol, we demonstrated that, by virtue of a cryptic mitochondrial leader sequence exposed by the truncation, the final destination of tCB is the mitochondrion (24), whereby nuclear fragmentation and cell death are elicited (13). Preliminary evidence for mitochondrial processing of the imported enzyme was provided by Western blots of cellular fractions separated by differential centrifugation (24). Although mitochondria possess the necessary machinery to fold imported proteins (25), we could not demonstrate any enzymatic activity of mitochondrial tCB thus far. In line with a hypothesis put forward by Sivaraman et al. (11) in analogy with cathepsin X, we can speculate that Cys 42p of the shortened proregion of truncated cathepsin B might form a transient disulfide bridge with the active site cysteine residue. However, proteolytic activity is not a stringent requirement for truncated cathepsin B to elicit pathophysiological effects in cells. In fact, certain proteolytic enzymes exert important physiological activities independent of their enzymatic activity. Thus, the leukocyte serine peptidases elastase, myeloblastin, and cathepsin G kill bacteria without involvement of their powerful catalytic centers (26, 27). For myeloblastin, a hysteretic mechanism aimed at preventing proteolytic activity during phagocytic killing of bacteria has been proposed (28, 29). These observations will be considered in future studies aiming at elucidating the physiological function of truncated cathepsin B.

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