PhD Thesis

ABSTRACT

REGULATION OF CATHEPSIN D ACTIVITY AND ACTIVATION

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Introduction

Cathepsin D (CD) is an aspartic peptidase located in the lysosomes of all mammalian cells, its main role is catabolic degradation of proteins. More over CD is known to participate in a range of physiological processes such as apoptosis and tissue homeostasis, as well as in the regulation of angiogenesis and the production of peptidic antigens. The role of CD in pathophysiology is associated with several diseases such as Alzheimer's disease and cancer.

Alzheimer's disease is a neurodegenerative disorder, which is generally considered to be the most common form of dementia. Progression of this disease is accompanied with the deposition of amyloid plaques (AP) in the brain, which leads to neurodegeneration. The AP is a fragment released from amyloid precursor protein (APP) cleaved by secretases[1]. High levels of CD were found in cerebrospinal fluid of the Alzheimer's patients[2]. It was demonstrated that CD is able to cleave APP and produce the pathogenic AP. A genetic polymorphism in the CD gene was reported, which changes single amino acid in the propeptide of CD zymogen. Some studies have shown that this variation is a major risk factor for the development of Alzheimer’s disease in some families or populations, however biochemical function of this substitution is unknown[4].

Procathepsin D (proCD), zymogen of CD, is overexpressed and hypersecreted by cancer cells where its concentration is correlated with increased metastatic potential[5]. Different mechanisms have been proposed as to be responsible for the mitogenicity of proCD. Some studies indicate its action as a ligand involved in interaction with unknown cell surface receptor. This interaction seems to be mediated by activation peptide of proCD [6]. On the other hand, activated forms of proCD has been implicated in degradation of extracellular matrix, activating growth factors or preventing secretion of growth inhibitors[7]. The question remains as to whether secreted proCD could be activated extracellularly and proteolytically active in a sufficiently acidic environment[7].

ProCD is used as an independent prognostic factor in clinical oncology, its increasing level in serum of patients indicates poor survival or relapse of the cancer[8]. It was suggested that immunization by proCD can prevent development of cancer, because antibodies against proCD can help to control cancer cells growth[9].

Aims of the thesis

This thesis is focused on human CD. Activation of proCD zymogen to CD and factors involved in regulation of this process were investigated by biochemical methods. The partial aims of this study are:

- Develop isolation procedure to obtain purified a) CD from mammalian tissues and b) proCD produced by transgenic human cell line.
- Analyze structure-function relationships in the activation peptide (propeptide) of proCD and to map interaction domains with the enzyme core.
- Determine factors like pH, sulphated polysaccharides and other processing peptidases involved in the regulation of proCD activation.

Results

Preparation of cathepsin D and its zymogen

The new effective isolation procedure of mature CD was developed. The protein was purified from human placenta which is nowadays the most available human organ.

The new purification procedure of proCD secreted by transgenic cells to medium was designed. The purification protocol utilizes an immunoaffinity chromatography at neutral pH. These conditions allow to obtain pure zymogen free of contamination by the autoactivation intermediate.

Modulation of cathepsin D activity by sulphated polysaccharides

It was reported previously that some sulphated polysaccharides (SPs) might modulate activity and stability of cysteine cathepsins[10]. So far the action of SPs on aspartic cathepsins was not yet investigated.

Results of this thesis show that SPs such as dextran sulphate, chondroitin sulphate and heparin, enhance proteolytic activity measured with peptidic and as well as protein substrates. This effect is dependent of pH, concentration and type of SPs. These macromolecules affect binding of substrate into the active site of CD which is demonstrated by significant decrease of K_m value for a substrate. CD is proteolytically active at acidic pH, in the presence of heparin the activity range extends towards the neutral pH conditions. Heparin-like glycosaminoglycans are presented on the cell surface and the extracellular matrix where are involved in a variety of biological functions[11]. This indicates that the presence of glycosaminoglycans might facilitate proteolytic action of CD in the extracellular milieu.

Structure-function analysis of procathepsin D propeptide

The propeptide (activation peptide) is located at the N-terminus of the proCD molecule, it blocks the entire active site and hence inhibits the enzyme activity. The structure of proCD has not yet been determined and neither was the mechanism of the propeptide action or its structure-function relationships.

A spatial model of proCD was constructed as a tool for structure-based design of peptidic fragments derived from its propeptide and adjacent N-terminus of the mature CD. The inhibitory potency of synthetic propeptide fragments was evaluated using the kinetic assay with the mature CD. These mapping indicates three structure domains interacting with the enzyme core: N-terminus of the propeptide, "Lys-Tyr anchor" at the active site, and N-terminus of the mature CD (Figure 1).

1) **The N-terminus of the propeptide** displays nanomolar inhibition, it interacts with enzyme core outside of the active site mainly through electrostatic interactions, which are sensitive to decreasing pH or presence of SPs.

2) Second inhibition domain (**"Lys-Tyr anchor"**) identified in the propeptide contains amino acids Lys34p-Tyr35p that interact through hydrogen bonds with catalytic aspartates. Inhibition of this domain is in the micromolar range and is weakened with decreasing pH. A genetic polymorphism in this segment was reported (major Ala38p, minor Val38p); it is assumed that this substitution is a major risk factor for the development of Alzheimer's disease in some families or populations[4]. Analysis of this position performed in this thesis indicates, that alanine in position 38p is necessary for effective interaction of entire domain with the enzyme core. It suggests that substitution of Ala38p by valine might modulate the activation of proCD.

3) **N-terminus of mature CD** serves as autoregulatory domain. At low pH, this segment is situated outside of the active site. At neutral pH, the mature N-terminus is repositioned into the active site and blocks access of the substrate.

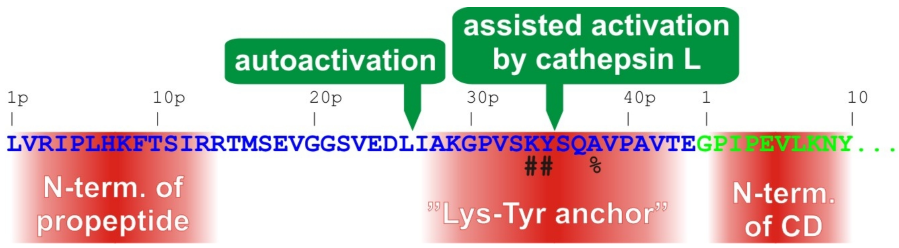

Figure 1. Positions of inhibitory domains identified in the propeptide and N-terminus of mature cathepsin D (CD). *Sequence of the propeptide is in blue (1p-44p), sequence of the N-terminus of the mature CD is in green (1-10). Inhibitory domains are highlighted in red boxes. Amino acids Lys34p and Tyr35p (#) of the propeptide interacts with catalytic aspartates. Substitution of Ala38p (%) to valine was reported to be associated with pathology. Green arrows point at bonds cleaved during autoactivation of proCD and activation assisted by cathepsin L. The suffix "p" indicates propeptide numbering.*

Activation of procathepsin D

This thesis presents the first detailed biochemical study of two mechanisms of proCD activation, namely autoactivation and assisted activation by cathepsin L.

The autoactivation takes place at acid pH and is associated with the removal of the N-terminus of the propeptide, which results in the formation of proteolytically active form called pseudoCD (Figure 2). The autoactivation process is accelerated in the presence of SPs. Moreover, the pH profile of proCD autoactivation is modulated by heparin that facilitates the autoactivation processing at close to neutral pH. In this way, the proCD secreted by cancer cells might be activated in the extracellular environment that is rich in heparin-like glycosaminoglycans. Such a glycosaminoglycans-mediated modulation of activation and activity of proCD/CD molecules can play an important role in the cancerogenesis process.

The results of this study suggest that the proCD activation represents a complex multi-pathway process (Figure 2). It was demonstrated that cathepsin L is able to proteolytically process proCD by cleaving its propeptide at the C-terminus (Figure 1). The product of this assisted activation termed pseudo(L1)CD is a new activation intermediate of proCD (Figure 2). This activation pathway takes place at neutral pH where the autoactivation does not occur.

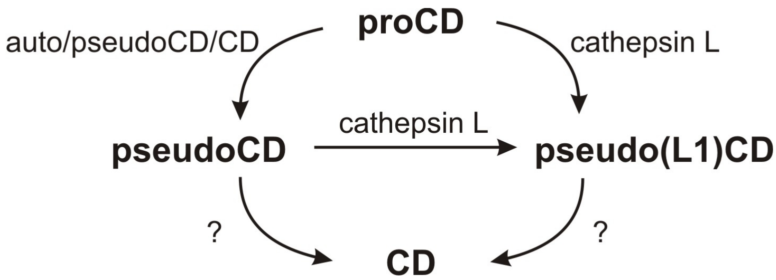

Figure 2. Scheme of activation of procathepsin D (proCD) to mature cathepsin D (CD). *Arrows represent events of proteolytic conversion; peptidases involved in this processing are indicated; "auto" means monomolecular autoactivation of proCD; "?" means unknown endo- or aminopeptidases involved in the generation of mature CD.*

Conclusion

In this work, biochemical mechanisms regulating the process of proCD activation were described. They are potentially involved in the conversion of proCD to mature CD, which represents an important event in a number of physiological and pathophysiological processes. It was demonstrated that SPs modulate the activity of mature CD and the activation of proCD, and a new activation intermediate pseudo(L1)CD was characterized. The structure-function mapping of the proCD propeptide identified inhibitory domains that can be used in the construction of inhibitors of CD and other medically important aspartic peptidases.

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