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ScienceDirect
Molecular Immunology 45 (2008) 2531–2536
Molecular Immunology
www.elsevier.com/locate/molimm

Activation of cathepsin L by the cathelin-like domain of protegrin-3

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Received 20 December 2007; received in revised form 5 January 2008; accepted 8 January 2008
Available online 4 March 2008

Abstract

The cathelin-like domain (CLD) of the antimicrobial cathelicidin family constitutes a unique protein family with structural similarity to cystatins, the cysteine protease inhibitors. CLDs are derived from the processed amino-terminal prosequence of the cathelicidin precursors with conservation across the vertebrate lineage ranging from fish to human. Initial attempt to characterize a possible inhibitory activity of protegrin-3 (PG3) CLD protein (a member of the multigene family of porcine cathelicidins) against several proteases led to an unexpected finding that PG3 CLD efficiently activated rather than inhibited human cathepsin L. Partial deletion of the L2 loop of PG3 CLD, a structurally equivalent region important in interaction of cystatins with proteases, significantly decreased its activating effect on cathepsin L. A complex model based on this functional loop was proposed to explain this unexpected effect, in which evolutionary emergence of completely opposite biological activity could be associated with structural discrepancies of the loop due to sequence variations between pig and human. Our results provide new insights into deeper understanding of the immune-related biological activity of this so-called pro-domain of the cathelicidin family.

Keywords: Innate immunity; Antimicrobial peptide; Cystatin; Cathelicidin

1. Introduction

Cathelicidins are a family of bipartite effector molecules of innate immunity identified by a substantial heterogenic carboxyl-terminal antimicrobial domain of 12–100 residues linked to an evolutionarily conserved amino-terminal cathelin-like domain (CLD) of approximately 99–114 residues. This heterogeneity is reflected by their structural diversity that includes all three major folding types of antimicrobial peptides (Tomasinsig and Zanetti, 2005; Zaiou and Gallo, 2002; Zanetti, 2005). Since the initial discovery in bovine, a large number of cathelicidins have been characterized from an array of phylogenetically distant vertebrates (Tomasinsig and Zanetti, 2005; Uzzell et al., 2003; Zaiou and Gallo, 2002; Zanetti, 2005).

The cathelicidin gene is translated as a precursor in the cytoplasmic granules of neutrophil/polymorphonuclear leukocytes (PMN) with a signal peptide removed during translation to yield the proform with two domains including the CLD and the antimicrobial domain. During the inflammatory response, the proform will further be processed to make functionally active by protease cleavage of the antimicrobial domain that will be released to sites of microbial infection. The proform isolated from the rabbit PMN *in vitro* exhibits high affinity to bind *Escherichia coli* and modulate the antibacterial actions of other leukocyte proteins on this Gram-negative bacterium (Zarember et al., 1997). Recent literature reported that some proforms can be mapped onto the cell surface of PMN. For example, a 15 kDa proform derived from a porcine cathelicidin was found to be associated with FcγRIIIaα on the cell surface (Sweeney and Kim, 2004), whereas the human proform hCAP-18/LL37, a well-characterized component of PMN-specific granules, was characterized to be translocated to the human PMN surface after the chemoattractant fMLF stimulation (Stie et al., 2007).

Although extensive studies have focused on the antimicrobial domains of the cathelicidin family due to their central roles in both innate and adaptive immunity through direct antimicrobial activity and as immune modulators and mediators of inflammation, the body of evidence for their possible immune-related

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doi:10.1016/j.molimm.2008.01.007

defense functions of CLDs has been growing in recent years. For instance, Zaiou et al. (2003) demonstrated that human hCAP-18/LL37 CLD was able to inhibit protease activity of cathepsin L and exhibited clear toxicity against both Gram-positive and -negative bacteria. Such inhibitory activity on cathepsin L could be associated with its structural similarity to type 2 cystatins which belong to secreted natural inhibitors of family C1 (papain-like) cysteine peptidases (Dieckmann et al., 1993). Given the key role of cathepsin L in antigen presentation (Honey and Rudensky, 2003), it is possible that the regulation of its activity by CLD can establish a link between innate and adaptive immunity, which will undoubtedly provide new insights into more understanding of specific and independent functions of CLD in host defense.

Here, we report an unexpected activating effect of porcine PG3 CLD which is completely contrary to its human counterpart hCAP-18/LL37. Mutational experiments combined with a structure complex model allow us to correlate this activity to a structurally flexible loop of PG3 CLD which could be involved in a direct interaction with cathepsin L. Biological significance of the activating effect on cathepsin L has been discussed in the context of antigen presentation.

2. Materials and methods

2.1. Construction of the CLD mutant (CLD-M)

To generate the mutant of PG3 CLD with seven residues in the L2 loop deleted, we designed a pair of back-to-back primers (FP: 5′-ATCACCTGCAATGAGGTTCAAGGT-3′; RP: 5′-ATCCAGGGTGACTGTCCCCACACA-3′) to perform inverse PCR amplification of the plasmid pET-15b-ProS (Sanchez et al., 2002). Primers FP and RP, respectively correspond to the amino acid sequences of ITCNEVQG and CVGTVTLD of PG3 CLD. Phosphorylation of FP and RP was carried out by T4 polynucleotide kinase and ATP (Takara, Dalian). PCR components include: 14 μl ddH_2O; 2 μl 10× Ex Taq buffer; 1 μl 10 mM dNTPs; 1 μl 5 μM kinased FP; 1 μl 5 μM kinased RP; 1 μl pET-15b-ProS [0.1 ng/μl]; 0.25 μl TaKaRa Ex Taq. Subsequently, the linear PCR product was circularized by T4 DNA ligase after end polishing using pfu polymerase and transformed into *E. coli* DH5α. Positive clone was confirmed by DNA sequencing and the plasmid pET-15b-ProS-m was transformed into *E. coli* BL21 (DE3) for protein expression.

2.2. Expression and purification of recombinant proteins

We used the similar method described by Sanchez et al. (2002) with some minor modifications to express and purify both CLD-M (mutant) and CLD-W (wild type). For the detailed description of the expression and purification methods, see Supplemental material 2. Protein concentration was determined according to the biuret method (Layne, 1957).

2.3. Analytical assays

The mass spectra were acquired on a time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex. with a nitrogen laser (337 nm). The samples were mixed in an eppendorf tube with the same volume of the matrix solution. A solution of a-CHCA was prepared at a concentration of 15 mg/ml in 2:1 (v/v) ACN/0.1% TFA. 1 μl of the mixtures were applied to a steel plate and introduced into the mass spectrometer after drying. The spectra were obtained in the linear mode by summing 200 laser shots with an ion source voltage 1 of 19 kV, ion source 2 of 16.27 kV. The instrument was calibrated externally by cytochrome *c* (Bruker). Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) at a protein concentration of 0.3 mg/ml dissolved in water. Spectra were measured at room temperature from 190 to 250 nm using a quartz cell of 1.0 mm path length. Data were collected at 1-nm intervals with a scan rate of 200 nm/min. The CD spectra measure was performed by averaging three scans. Secondary structure content was estimated by JASCO CD standard analysis.

2.4. Protease activity assays

Protease inhibitory activities of recombinant CLDs were assayed with fluorescence-conjugated casein substrate (EnzCheck® Protease Assay Kit green fluorescence, #E-6638, Invitrogen) by measuring their inhibitory action against human liver cathepsin L (#219402, Calbiochem, CA, USA), human neutrophil elastase (#324681, Calbiochem), bovine pancreas trypsin (#T1426, Sigma–Aldrich, MO, USA). Protease assay reaction mixture includes: 100 μl of FL-conjugated casein substrate; 97 μl of buffer; 2 μl of 100 μM PG3 CLD-W or CLD-M samples (final concentration 1 μM) and 1 μl of proteases. Cathepsin L (0.1 or 0.05 mU) in the assay buffer (340 nM sodium acetate, 60 mM acetic acid, 8 mM dithiothreitol, and 4 mM EDTA, pH 5.2), elastase (2 or 0.2 mU) in 20 mM Tris (pH 7.8), or trypsin (0.5 μg or 50 ng) in 20 mM Tris (pH 7.8) were preincubated for 2 min at RT with cathelin-like protein (PG3 CLD-W or PG3 CLD-M, at designated concentration) before adding BODIPY FL casein substrate. Reaction mixtures were incubated at 37 °C for designated time periods, and protease activity was monitored as fluorescence level with SpectraMax GEMINI EM (Molecular Devices Corporation, Sunnyvale, CA). Statistical analyses were done by two-way ANOVA with Graphpad PRISM 4 (Graphpad Software Inc.).

3. Results and discussion

3.1. Characterization of recombinant CLD-W and CLD-M

CLD-W and CLD-M were expressed in *E. coli* as His-tagged proteins (Fig. 1(A)) which were purified on a nickel column. After the removal of His-tag and further purified by rp-HPLC (Supplemental material 1), recombinant CLD-W and CLD-M were used for analytical assays. Molecular weights (MWs) of purified products were determined by MALDI-TOF, which, respectively gave 11719.49 and 10909.32 Da for CLD-W and CLD-M, highly consistent with their theoretical MWs of 11718.23 and 10908.31 (Fig. 1(B)). CD spectra of CLD-W and CLD-M shown in Fig. 1(C) are similar each other, characterized by a minimum at 205 nm and a shoulder in the 222–225 nm