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ScienceDirect
Molecular & Biochemical Parasitology 150 (2006) 318–329
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Differentiation of *Leishmania major* is impaired by over-expression of pyroglutamyl peptidase I

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Received 6 July 2006; received in revised form 6 September 2006; accepted 7 September 2006
Available online 27 September 2006

Abstract

Pyroglutamyl peptidases I (PPI) are cysteine peptidases of the clan CF, family C15, which hydrolyse N-terminal L-pyroglutamyl residues (L-pGlu). The L-pGlu modification is a post-transcriptional modification that confers relative aminopeptidase resistance and, in some cases, is essential to the modified peptides' biological activity. PPIs have been identified in a variety of organisms, although definitive biological functions have yet to be attributed to them. The *L. major PPI* was expressed in *Escherichia coli* as active recombinant enzyme, and shown to have biochemical properties more similar to mammalian than bacterial PPIs. The LmPPI active site catalytic triad of E101, C210, and H234 was confirmed by mutagenesis. PPI activity was detected in *L. major* promastigotes, and the enzyme localised to the parasite cytosol. No detectable phenotype could be observed for *L. major PPI*-deficient mutants, which retained infectivity to macrophages in vitro and mice. However, over-expression of the active PPI, but not inactive PPI^{C210A}, in *L. major* impaired differentiation from the procyclic promastigote to the infective metacyclic promastigote. Susceptibility to a natural L-pGlu-modified antimicrobial peptide, gomesin, was tested using the different cell lines, which were all equally susceptible. Whilst PPI is widespread through the eukaryotic kingdom, this study now suggests that the enzyme is not essential for normal eukaryotic cell function. However, PPI could be involved in regulating the action of L-pGlu-modified peptides required for differentiation of *L. major*.

Keywords: *Leishmania*; Parasite; Pyroglutamyl peptidase I; Differentiation

1. Introduction

Pyroglutamyl peptidase (also referred to as pyrrolidonecarboxylate peptidase, pyroglutamate aminopeptidase, pyrrolidonecarboxylyl peptidase, 5-oxoprolyl peptidase, pyroglutamyl aminopeptidase and pyroglutamyl peptide hydrolase) belongs to the class of omega peptidases (EC 3.4.19.−) and hydrolyses N-terminal L-pyroglutamate residues (L-pGlu) from peptides and proteins containing this modification [1]. Pyroglutamyl peptidase activity was first detected in *Pseudomonas fluorescens* [2] and among the organisms it has been since described in are mammals [1,3], bacteria [4,5], reptiles [6], and recently, trypanosomes [7]. They are divided into two classes, the cysteine peptidase pyroglutamyl peptidase I (EC 3.4.19.3., MEROPS clan CF, family C15), and the metallopeptidase pyroglutamyl peptidase II (EC 3.4.19.6., MEROPS clan M01, family M1). Pyroglutamyl peptidase I is a soluble, intracellular enzyme [8], whereas pyroglutamyl peptidase II is membrane-bound [9].

Mammalian pyroglutamyl peptidase I (PPI) occurs as a monomeric enzyme of relatively low molecular mass (∼25 kDa), and with a pH optimum from 6.5 to 8.5, that is located in the cytosol in mammals [1,3]. Although PPI has been shown to have broad substrate specificity, it also has a strong specificity for L-pGlu residues and will not cleave modified L-pGlu residues [10]. The catalytic triad, determined as E81, C144 and H168 for PPI of *Bacillus amylotumefaciens* [4], is conserved in the human and the mouse enzymes [3].

Abbreviations: kb, kilobase(s); bp, base pairs; *PPI*, gene encoding pyroglutamyl peptidase I; PPI, protein encoded by *PPI*; rLmPPI, recombinant *L. major* PPI; PCR, polymerase chain reaction; RT PCR, reverse transcriptase PCR; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenylmethylsulfonyl fluoride; TCEP, Tris (2-carboxyethyl) phosphine

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doi:10.1016/j.molbiopara.2006.09.004

The biological activity of some peptides is regulated in part via cyclisation of N-terminal glutamine to L-pGlu by the enzyme glutaminyl cyclase. This cyclisation confers a relative stability towards aminopeptidase degradation. Notably, 12.3% of proteins of the NCBI RefSeq database with signal peptides are initiated with Gln once the signal peptide is removed [11]. Also, removal of the N-terminal translation initiator methionine is often crucial for the function and stability of proteins [11,12], and if the second amino acid is a Gln then a L-pGlu-modified peptide can be generated [13]. How many such candidates for pyroglutamylation are so modified is unknown. Among naturally occurring peptides known to have L-pGlu modification are neuropeptides. Moreover, the L-pGlu N-terminal modification can be a determinant in biological activity of peptides [14] and so PPI has been attributed a role in neuropeptide regulation and metabolism in mammalian systems. However, the lack of its release from the cells still causes doubts regarding its function [15]. PPI may participate in protein metabolism [16], but very few mammalian PPIs have been fully characterised and a definite physiological role still has to be attributed.

Most bacterial PPIs examined so far are soluble, have broad substrate specificity, require a reducing environment and occur as multimers [17,18]. In terms of substrate specificity, catalytic residues and inhibitor susceptibility, they resemble the mammalian PPI enzymes. Moreover, as with mammalian PPIs, a definite function still has to be attributed. It has been suggested they may play a role in intracellular protein metabolism [19], though the presence of pyroglutamyl peptidase activity is not ubiquitous among the bacterial kingdom.

In addition to neuropeptides, various bioactive peptides possess a N-terminal L-pGlu modification and are synthesised by various organisms. As examples, a variety of antimicrobial peptides have been isolated from mammals [20], insects [21], shrimp [22], and spiders [23]. The widespread occurrence of these compounds suggests they may play a role in innate immunity against micro-organisms and other pathogens [24,25]. Gomesin, an 18 amino acid N-terminally L-pGlu-modified peptide isolated from a spider [23] showed strong bacterial growth inhibition, affected development of filamentous fungi and yeast, and also was toxic to *Leishmania amazonensis*. This observation stimulates the hypothesis that some bacteria and protozoan parasites may possess a PP activity as protection against such bioactive compounds they encounter. There is some evidence that the innate immune response of humans involves antimicrobial peptides. Humans possess two classes of defensins, α- and β-defensins, which are cationic 15–45 amino acid long peptides with a pattern of disulfide bridges essential to the activity [26]. Some of the β-defensins possess a L-pGlu modification at the N-terminus [27]. In insects, various homologues of the defensins exist, namely the cecropins [28]. Reports of toxicity of human antimicrobial peptides on parasitic protozoa are scarce. The parasite *Giardia lamblia* can be killed by cationic neutrophil peptides produced by the intestine epithelium [29], and that resistance of humans against *Trypanosoma brucei* may be aided by antimicrobial peptides in the serum [30]. Interestingly, it was recently reported that this parasite releases PPI which degrades serum neuropeptides [7], suggesting that this enzyme may also provide some protection against the antimicrobial peptides. Moreover, an antimicrobial peptide with a N-terminal L-pGlu has been identified in *Drosophila* [31]. Thus there is some evidence that L-pGlu-modified peptides with antimicrobial activity exist in mammals and insects, the two hosts of *Leishmania*.

This study focused on the PPI of *L. major* with the aim of understanding how the enzyme plays a part in the biology of the parasite and its interaction with its two hosts and so with the intention of discovering more on the occurrence and importance of L-pGlu-modified peptides. The results suggest a key role for such peptides in differentiation of *Leishmania*, but the data show that the PPI enzyme itself is not essential for parasite proliferation.

2. Materials and methods

2.1. Parasite material

Leishmania major (MHOM/IL/80/Friedlin) promastigotes were cultured in modified Eagle's medium (GibcoBRL, designated as HOMEM medium) supplemented with 10% (v/v) heat inactivated foetal calf serum (HIFCS) at 25 °C as described previously [32]. The required antibiotics were added to *PPI* knock-out and *PPI* over-expressing cultures as follows: hygromycin B (Sigma) at 50 μg/ml; blasticidin S (Calbiochem) at 15 μg/ml; and neomycin (G418, Geneticin, Life Technologies) at 50 μg/ml.

L. major metacyclic promastigotes were purified from stationary phase cultures as described [33]. Briefly, promastigotes were resuspended in phosphate buffer saline (PBS) at 10^8 cells/ml. Peanut agglutinin (Vector Laboratories) was added at 50 μg/ml and the sample was incubated 25 min at room temperature. The supernatant contained the non-agglutinated metacyclic promastigotes. *L. major* amastigotes were purified from infected BALB/c mice as described [34].

Parasites lysates were obtained by resuspension in 50 mM Tris–HCl pH 8.0, 0.25% (v/v) Triton X-100, 20% (v/v) glycerol, and, unless stated otherwise, a mixture of peptidase inhibitors (10 μM E-64, 2 mM 1,10-phenanthroline, 4 μM pepstatin A, 1 mM PMSF). Lysates were centrifuged at 13,000 × *g* for 15 min at 4 °C. The supernatant (designated as the soluble fraction) was separated from the pellet, and the pellet (designated as the membrane-bound fraction) was resuspended in lysis buffer. Protein concentrations were determined using the Bradford assay (Bio-Rad).

2.2. Bioassay for leishmanicidal activity

Promastigote viability was measured using the MTT assay as described [23]. 5×10^6 *L. major* promastigotes were incubated with gomesin and gomesin analogues at various concentrations. After an incubation for 1 h at 25 °C, MTT was added to a final concentration of 1 mg/ml, the mixture was incubated for 1 h at 25 °C, and the absorbance at 620 nm was read. Gomesin and gomesin analogues were synthesised as described [23]. The primary amino acid structure of gomesin is ZCRRLCYKQRCVTYCRGR* (using the single letter amino acid code, were Z stands for L-pGlu and the asterisk indicates