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Research paper

Antibacterial and leishmanicidal activities of temporin-SHd, a 17-residue long membrane-damaging peptide

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ABSTRACT

Temporins are a family of short antimicrobial peptides (8–17 residues) that mostly show potent activity against Gram-positive bacteria. Herein, we demonstrate that temporin-SHd, a 17-residue peptide with a net charge of +2 (FLPAALAGIGGILGKLF$_{amide}$), expressed a broad spectrum of antimicrobial activity. This peptide displayed potent antibacterial activities against Gram-negative and Gram-positive bacteria, including multi-drug resistant *Staphylococcus aureus* strains, as well as antiparasitic activity against promastigote and the intracellular stage (amastigote) of *Leishmania infantum*, at concentration not toxic for the macrophages. Temporin-SHd that is structured in a non-amphipathic α-helix in anionic membrane-mimetic environments, strongly and selectively perturbs anionic bilayer membranes by interacting with the polar head groups and acyl region of the phospholipids, with formation of regions of two coexisting phases: one phase rich in peptide and the other lipid-rich. The disruption of lipid packing within the bilayer may lead to the formation of transient pores and membrane permeation/disruption once a threshold peptide accumulation is reached. To our knowledge, Temporin-SHd represents the first known 17-residue long temporin expressing such broad spectrum of antimicrobial activity including members of the trypanosomatidae family. Additionally, since only a few shorter members (13 residues) of the temporin family are known to display antileishmanial activity (temporins-TA, -TB and -SHa), SHd is an interesting tool to analyze the antiparasitic mechanism of action of temporins.

1. Introduction

Temporins constitute a large family of α-helical antimicrobial peptides (AMPs) that are produced by the skin of Eurasian and New World ranid frogs [1–5]. General characteristics of temporins include a small size (8–17 amino acids), a low net positive charge from 0 to +3, and a highly variable sequence with a prevalence of hydrophobic amino acids. Most naturally occurring temporins contain a single basic amino acid residue and show potent antimicrobial activity against Gram-positive bacteria [2–4], but are inactive or weakly active against Gram-negative bacteria. Only temporin-SHa (FLSGIVGMLGKLF$_{amide}$) from *Pelophylax saharicus* [6], as well as temporin-Tl (FVQWFSKFLGRIL$_{amide}$) from *Rana temporaria* [7] and temporin-1DRa (HFLGTLVNLAKKIL$_{amide}$) from *Rana draytonii* [8] that bear a net charge of +3, exhibit potent and very broad spectra of activity against Gram-positive and Gram-negative bacteria and yeasts. Despite their short length and low net charge, temporins are able to disrupt anionic (prokaryotic) membranes in a similar way and to a similar extent of that of most highly positively charged long AMPs. Only a few temporins, temporins-Ta and -Tb from *Rana temporaria*, and temporin-SHa have also leishmanicidal activity at concentrations that are not toxic to macrophages [6,9]. Temporins form amphipathic α-helices with alternating hydrophobic and polar residues in apolar media or membrane mimetic environments [9–13]. The amphipathic helical structure enables

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these cationic peptides to interact with and to destabilize the bacterial cytoplasmic membrane, thereby provoking membrane permeabilization and/or disruption via a "carpet-like" mechanism [10–12,14,15].

Antimicrobial activities of AMPs are mediated by a complex and sensitive balance between various interrelated peptide parameters including net charge, hydrophobicity, amphipathicity, degree of structure formation and flexibility [16–22]. Although known naturally occurring AMPs differ dramatically in size (from 8 to 100 amino acids), the effects of length on antimicrobial activity, selectivity and mechanism of action have been only scarcely evaluated [23–27]. In that regard, new members of the temporin family comprise of up to 17 amino acids have been recently characterized in extracts of the skin of various species of ranids [28–32] (Fig. 1B). However, their activities against bacteria, fungi and protozoa have not been well documented and their mechanisms of action remain unknown. These long-temporin peptides represent good models to be compared with short 13-residue temporins, in order to pinpoint the effects of length on the microbicidal activity and the mode of action of this class of peptides, and to evaluate the role(s) of the remainder of the residues in the structure and membrane-disturbing activity of the bound peptides.

Peptides of the temporin family belong to the dermaseptin superfamily of host defense peptides produced by the skin of ranid and hylid frogs [33]. Peptides of the dermaseptin superfamily are genetically related, with a remarkable identity in signal sequences and intervening sequences of their pre-proforms [34,35]. We have recently used the conservation of the pre-proregion sequences of the preprodermaseptin transcripts to identify a new 17-residue long member of the temporin family (Fig. 1), named temporin-SHd (Temp-SHd), in the North African ranid frog *P. saharicus* (family: Ranidae) [36]. Herein, a detailed characterization of the activities, structure and membrane-damaging properties of synthetic temporin-SHd was undertaken using i) antimicrobial assays against Gram-positive bacteria, Gram-negative bacteria, yeasts, fungi, and the human protozoan parasite *Leishmania* ii) bacterial permeation assays iii) circular dichroism and iv) differential scanning calorimetry on multilamellar vesicles composed of anionic and zwitterionic lipids as models for prokaryotic and eukaryotic plasma membranes.

2. Materials and methods

2.1. Purification and identification of Temp-SHd in the frog skin of *P. saharicus*

We have previously cloned the Temp-SHd precursor from the skin (130 mg) of one adult specimen of *P. saharicus* frog (Béja, Tunisia) using specific primers matching the conserved signal peptide-coding region and the 3′-UTR of temporin precursors [36]. In order to characterize the mature peptide, acidic extraction was performed from the frog skin at 4 °C by homogenization in 10% acetic acid using a polytron homogenizer. After centrifugation (10,000 × g, 20 min, 4 °C) of the homogenate to remove insoluble material, the supernatant was lyophilized. The powder was dissolved in 0.1% trifluoroacetic acid (TFA)/H_2O, centrifuged (13,000 × g, 10 min, 4 °C), filtered (0.45 μm) and lyophilized again. For the identification of Temp-SHd, 40 mg of this skin powder were dissolved in 0.1% TFA/H_2O, centrifuged (13,000 × g, 10 min, 4 °C)

A

ppTemp-SHd

```
F  L  G  T  I  N  L  S  L  C  E  Q  E  R  D  A  D  E  E  K
TTCCTTGGGACCATCAACTTATCTCTCTGTGAGCAAGAGAGAGATGCCGATGAAGAAAAA   60

R  D  E  P  D  E  S  D  V  E  V  E  K  R  F  L  P  A  A  L
AGAGACGAGCCAGATGAAAGTGATGTTGAAGTAGAAAAACGATTTTTACCAGCAGCCCTT  120

A  G  I  G  G  I  L  G  K  L  F  G  K  *
GCAGGTATTGGAGGAATTTTGGGTAAACTTTTCGGCAAATAACCCAAAAATGTTGAAACT  180

TTGGAAATGGAATTGGAAATCATCTGACGTGGAATATCATTTGGCTAAATGCTCAACAGA  240
TGTC                                                           244
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B

		Net charge	<H>	<μH>
Temp-SHd	FLPAALAGIGGILGKLFa	+ 2	0.86	0.51
Temp-LTa	FFPLVLGALGSILPKIFa	+ 2	1.04	0.47
Temp-ERa	FLPLIIGALSSLLPKIFa	+ 2	1.06	0.49
Temp-LT2	FLPIALKALGSIFPKILa	+ 3	0.93	0.38
Temp-GUc	FFPLIFGALSSILPKILa	+ 2	1.07	0.49
Temp-SKa	FLPVILPVIGKLLNGILa	+ 2	1.05	0.55
Temp-SKb	FLPVILPVIGKLLSGILa	+ 2	1.09	0.52

Fig. 1. (A) Partial nucleotide sequence and deduced amino acid sequence of the cDNA encoding pre-protemporin-SHd (ppTemp-SHd) cloned from the skin of *Pelophylax saharicus* [36] (EMBL accession number: AM903075). The partial putative signal peptide (gray) is followed by the acidic propiece and the mature peptide (black). The G residue (in bold) at the C-terminal end of the mature temporin sequence serves as an amide donor after removal of the K residue (in bold) with a carboxypeptidase. The stop codon (asterisk) and the 3′-UTR sequence (italic) are indicated. (B) Comparison of the primary structures and physicochemical properties of 17-residue long temporins characterized in the skins of frogs from the genera *Pelophylax* (Temp-SHd), *Hylarana* (Temp-ERa, Temp-LTa, Temp-LT2) [28,32], and *Rana* (Temp-GUc, Temp-SKa, Temp-SKb) [29,31]. The shaded residues are conserved between species. The basic residues are indicated in bold. a, amide. <*H*>: mean hydrophobicity; <*μH*>: mean amphipathic moment [54].