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An orthologue of the host-defense protein psoriasin (S100A7) is expressed in frog skin

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ABSTRACT

Host-defense peptides and proteins are vital for first line protection against bacteria. Most host-defense peptides and proteins common in vertebrates have been studied primarily in mammals, while their orthologues in non-mammalian vertebrates received less attention. We found that the European Common Frog *Rana temporaria* expresses a protein in its skin that is evolutionarily related to the host-defense protein S100A7. This prompted us to test if the encoded protein, which is an important microbicidal protein in human skin, shows similar activity in frogs. The *R. temporaria* protein lacks the zinc-binding sites that are key to the antimicrobial activity of human S100A7 at neutral pH. However, despite being less potent, the *R. temporaria* protein does compromise bacterial membranes at low pH, similar to its human counterpart. We postulate that, while amphibian S100A7 likely serves other functions, the capacity to compromise bacterial cell membranes evolved early in tetrapod evolution.

1. Introduction

S100A7, also known as psoriasin, is a small protein that plays a key role in innate immune defense against bacteria in humans and other mammals (Gläser et al., 2005; Regenhard et al., 2009). The main target of human S100A7 is thought to be *Escherichia coli* (Gläser et al., 2005). The name psoriasin originates from its discovery in keratinocytes of patients suffering from the skin condition psoriasis, where this protein is overexpressed (Madsen et al., 1991). S100A7 is not only secreted by keratinocytes of the skin (Gläser et al., 2005; Madsen et al., 1991) but is also considered a principal *E. coli*-killing factor of the human tongue and the epithelium layer of the urogenital tract (Meyer et al., 2008; Mildner et al., 2010). Apart from being an important bactericidal protein, S100A7 also functions in skin differentiation, cancer, and as a chemotactic protein during inflammation (Hattori et al., 2014; Jinquan et al., 1996; Moubayed et al., 2007; Ye et al., 2013). Disulphide-reduced S100A7 acts as a broad-spectrum fungicide on human skin (Hein et al., 2015).

S100A7 is a member of the S100 protein family, a group of calcium-binding proteins involved in various intracellular and/or extracellular processes through interaction with specific target proteins. These processes include signal transduction, cell proliferation, differentiation, migration and inflammation. Additionally, S100 proteins inside the cell are important in the regulation of Ca^{2+} homeostasis and energy metabolism, while activities of S100 proteins outside of the cell include antimicrobial activity, tissue repair, and regulatory effects on inflammatory (and other) cells (Donato, 2003; Donato et al., 2013; Zimmer et al., 2005). Most S100 proteins have multiple functions. S100 proteins typically contain two EF-hand calcium-binding domains. However, S100A7 differs from the other S100 proteins in its N-terminal EF-hand, which probably does not bind calcium (Brodersen et al., 1998).

Although many antimicrobial agents act through permeabilization of the bacterial cell wall and membrane, no membrane damage was detected when *E. coli* were incubated at neutral pH with concentrations of human S100A7 above the minimal microbicidal concentration (Gläser et al., 2005), suggesting that the bacteria were killed through a different mechanism. Both preincubation of human S100A7 with Zn^{2+} ions and inactivation of the zinc-binding motif reduced or inhibited bactericidal activity, indicating that S100A7 acts through Zn^{2+} sequestration, resulting in death of *E. coli* because of zinc deprivation (Gläser et al., 2005; Lee and Eckert, 2007). Human S100A7 forms an antiparallel homodimer with two times four zinc binding residues that can bind two Zn^{2+} ions per dimer, using two histidine residues from one subunit and a histidine and aspartic acid residue from the other (Brodersen

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et al., 1999). Conversely, at pH 5.5, pore formation by human S100A7 has been detected both in liposomes and in *Bacillus megaterium* (Michalek et al., 2009), indicating that this protein can kill bacteria through different mechanisms depending on the pH. As the natural human skin pH is usually around 5 (Lambers et al., 2006), while the pH of the human tongue is slightly above 7 (Tolentino et al., 2011), the bactericidal activity of S100A7 in these two expression sites possibly relies on different processes.

Other S100 family members in human for which bacteriostatic and/or bactericidal activity has been shown, are S100A7A, S100A8, S100A9 and S100A12. S100A7A (also called S100A15) is only found in primates and apparently evolved by gene duplication from S100A7 during primate evolution (Wolf et al., 2011). Actually, genomic analysis revealed a series of recent duplications of the S100A7 gene leading to a total of five S100A7-like genes in the human genome, two of which are pseudogenes (Kulski et al., 2003). S100A8 and S100A9 show bacteriostatic and sometimes bactericidal activity *in vitro* upon formation of a heterodimer (S100A8/A9 or calprotectin) (Sohnle et al., 1991; Steinbakk et al., 1990) as a result of zinc and/or manganese sequestration (Damo et al., 2013; Kehl-Fie and Skaar, 2010; Sohnle et al., 1996; Yui et al., 1997). S100A8, S100A9 and S100A8/A9 complete various intracellular and extracellular functions in inflammation and innate immunity through chemotactic effects, antioxidant defense and the induction of apoptosis in vertebrate (tumor) cells (Gebhardt et al., 2006; Ghavami et al., 2008; Gomes et al., 2013; Ryckman et al., 2003). S100A12 homodimers act through zinc chelation, allowing this protein to participate in the control of *Helicobacter pylori* growth and virulence, and to inhibit the activity of matrix metalloproteinases (Goyette et al., 2009; Haley et al., 2015). S100A12 also possesses filaricidal and filaristatic activity, causes mast cell activation and is chemotactic for neutrophils, macrophages and mast cells (Gottsch et al., 1999; Miranda et al., 2001; Yang et al., 2007). Expression of S100A8/A9 and S100A12 in non-pathological circumstances is limited to leukocytes (Vogl et al., 1999) but can be induced in keratinocytes, endothelial cells, and epithelial cells during inflammation (Brandtzaeg et al., 1987; Kelly et al., 1989; Robinson and Hogg, 2000).

Genes encoding proteins of the S100 family are widespread in vertebrates, but S100A7 has not yet been reported outside amniotes. Up to now, only mammalian S100A7 proteins have been tested for their antimicrobial properties (Bruhn et al., 2011; Gläser et al., 2005; Lee and Eckert, 2007; Michalek et al., 2009; Regenhard et al., 2009; Teijeiro and Marini, 2015). While the antimicrobial potency of bovine S100A7 against *E. coli* is comparable to human S100A7, S100A7 from horse shows weak antimicrobial activity against *E. coli* and porcine S100A7 did not show microbicidal activity against *E. coli* at pH 7.4 (Bruhn et al., 2011; Regenhard et al., 2009; Teijeiro and Marini, 2015). We recently screened a cDNA library prepared from the skin of the European common frog *Rana temporaria* and found a transcript that encodes a protein of the S100 family. This gave us the opportunity to investigate whether the S100 protein family evolved bactericidal activity in amphibians. Because our phylogenetic analyses indicate that the encoded protein is orthologous to human S100A7, we refer to the *R. temporaria* protein as RtS100A7. Despite the absence of zinc-binding sites in RtS100A7, this protein is able to inhibit the growth of bacteria, suggesting that a zinc-independent antimicrobial activity in S100 proteins evolved early in tetrapod evolution.

2. Material and methods

2.1. Construction of a cDNA library

One male *Rana temporaria* was collected at Haacht, Belgium (March 29, 2011) and euthanized (Permit ANB/BL-FF/V11-00033) and total RNA was extracted from sampled skin tissue following the TRI Reagent protocol (Sigma-Aldrich). RNA was reverse transcribed and cloned into a vector using the Creator SMART cDNA library construction kit (Clontech). One Shot TOP10 Electrocomp *E. coli* electrocompetent cells (Invitrogen) transformed with cDNA library plasmids were grown overnight on LB/chloramphenicol plates (30 μg/ml final chloramphenicol concentration) and randomly picked colonies were amplified using vector-specific primers (M13-For: GTAAAACGACGGCCAGT; M13-Rev: AACAGCTATGACCATGTTCA). The PCR conditions for amplification were as follows: initial denaturation for 240 s (s) at 94 °C, followed by 25 cycles with denaturation for 40 s at 94 °C, annealing for 60 s at 55 °C, and elongation for 60 s at 72 °C. PCR products were purified (Qiagen PCR purification kit), amplified using the BigDye Terminator Sequencing Kit v.3.1 and sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequences were assembled into contiguous sequences using the CodonCode Aligner software (CodonCode Corporation). These contiguous sequences were compared to the database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). The cDNA sequence encoding RtS100A7 was deposited in GenBank under accession number KX343042.

2.2. Evolutionary analyses

A dataset was constructed containing RtS100A7 and representatives of all S100 protein family members of several mammals, amphibians and the zebrafish (*Danio rerio*). The protein sequences in this dataset were aligned using the G-INS-i algorithm implemented in Mafft (Katoh and Standley, 2013). Subsequently, phylogenetic relationships were estimated by Bayesian phylogeny inference using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) and maximum likelihood bootstrapping using RAxML 7.0.4 (Randomized AXelerated Maximum Likelihood) (Stamatakis, 2014; Stamatakis et al., 2008) on the CIPRES Science Gateway web server (Miller et al., 2010). The Bayesian phylogenetic analysis was executed with a mixed amino acid model. Two parallel runs of four incrementally heated Markov Chain Monte Carlo (MCMC) chains were performed (temperature parameter = 0.2), with a length of 10,000,000 generations, a sampling frequency of 1 per 1000 generations, and a burn-in corresponding to the first 2,500,000 generations. Convergence of the parallel runs was confirmed by split frequency standard deviations (less than 0.01) and potential scale reduction factors (approximating 1.0) for all model parameters, as reported by MrBayes.

Maximum likelihood bootstrap percentages for branch support were based on 500 bootstrap replicates using RAxML's fast hill-climbing algorithm, employing the amino acid substitution matrix WAG (Whelan and Goldman, 2001), to which the Bayesian analysis had converged, with gamma correction of among-site rate heterogeneity and estimation of the proportion of invariable sites.

2.3. Structural analyses

The presence of an N-terminal signal peptide in the encoded protein was investigated using SignalP 4.1 (Petersen et al., 2011). Protein domains were predicted using InterPro (protein sequence analysis & classification), which combines protein signatures from a range of different protein family databases (Mitchell et al., 2015). The structure prediction tool PSIPRED v3.3 was used to predict the secondary structure of the protein (Buchan et al., 2013; Jones, 1999). The binding sites for calcium and zinc in human S100A7 have been identified by Brodersen and coworkers (Brodersen et al., 1999,

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