Developmental and Comparative Immunology 35 (2011) 644–649

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Host defense peptides in skin secretions of the Oregon spotted frog *Rana pretiosa*: Implications for species resistance to chytridiomycosis

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ARTICLE INFO

Article history:
Received 17 December 2010
Received in revised form 18 January 2011
Accepted 24 January 2011
Available online 1 February 2011

Keywords:
Frog skin secretions
Antimicrobial peptides
Chytridiomycosis
Esculentin-2
Ranatuerin-2
Brevinin-1
Temporin

ABSTRACT

Population declines due to chytridiomycosis among frogs belonging to the Amerana (*Rana boylii*) species group from western North America have been particularly severe. Norepinephrine-stimulated skin secretions from the Oregon spotted frog *Rana pretiosa* Baird and Girard, 1853 were collected from individuals that had been previously infected with the causative agent *Batrachochytrium dendrobatidis* but had proved resistant to developing chytridiomycosis. These secretions contained a more diverse array of antimicrobial peptides than found in other species from the Amerana group and 14 peptides were isolated in pure form. Determination of their primary structures identified the peptides as esculentin-2PRa and -2PRb; ranatuerin-2PRa, -2PRb, -2PRc, -2PRd, and -2PRe; brevinin-1PRa, -1PRb, -1PRc, and -1PRd; and temporin-PRa, -PRb, and -PRc. The strongly cationic ranatuerin-2PRd and the esculentin-2 peptides, which have not been identified in the secretions of other Amerana species except for the closely related *R. luteiventris*, showed the highest growth inhibitory potency against microorganisms. The strongly hydrophobic brevinin-1PRd was the most cytotoxic to erythrocytes. Although no clear correlation exists between production of dermal antimicrobial peptides by a species and its resistance to fatal chytridiomycosis, the diversity of these peptides in *R. pretiosa* may be pivotal in defending the species against environmental pathogens such as *B. dendrobatidis*.

1. Introduction

The Amerana species group (also known as the *Rana boylii* group) of frogs from western North America traditionally comprised five species that were classified together on the basis of morphological criteria: *R. aurora*, *R. boylii*, *R. cascadae*, *R. muscosa*, and *R. pretiosa* (Hillis and Wilcox, 2005). Subsequently, *R. pretiosa* was divided into two species with distinct geographical ranges (*R. pretiosa* in the west and *R. luteiventris* in the east) (Green et al., 1997). More recently, the red-legged frogs, originally regarded as a single polytypic species with two subspecies the northern red-legged frog *R. aurora aurora* and the California red-legged frog *R. aurora draytonii*, have also been classified as separate species, *R. aurora* and *R. draytonii* that do not appear to be even sister taxa (Shaffer et al., 2004). Similarly, *R. sierra* from the Sierra Nevada of California and extreme western Nevada has been removed from the synonymy of *Rana muscosa* (Vredenburg et al., 2007). Molecular data based upon mitochondrial DNA sequences provide support for the monophyly of the Amerana and suggest that the group is 8 million years old (Macey et al., 2001). However, phylogenetic and evolutionary relationships among members of the group have yet to be fully resolved and any particular species may contain multiple divergent clades (Funk et al., 2008).

The near cosmopolitan emergence of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* has frequently led to catastrophic epizootics in frog populations (Fisher et al., 2009). This aquatic fungus parasitizes the mouthparts of larvae and the keratinized epidermis of post-metamorphic individuals which depletes electrolytes and impairs osmotic homeostasis leading to death (Voyles et al., 2009). Population declines due to fatal chytridiomycosis have been severe among frogs belonging to the Amerana species group. *B. dendrobatidis* infection has been detected and fatalities due to chytridiomycosis have been observed in every recognized species within the group [*R. aurora* (Nieto et al., 2007), *R. boylii* (Padgett-Flohr and Hopkins, 2009), *R. cascadae* (Garcia et al., 2006), *R. draytonii* (Padgett-Flohr and Hopkins, 2009), *R. luteiventris*

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doi:10.1016/j.dci.2011.01.017

(Pearl et al., 2007), *R. muscosa* (Fellers et al., 2001; Rachowicz et al., 2006), *R. pretiosa* (Hayes et al., 2009) and *R. sierra* (Morgan et al., 2007)].

The ability of a chytrid-infected animal to mount a cell-mediated or humoral adaptive immune response is poor (Rollins-Smith et al., 2009; Rosenblum et al., 2009; Stice and Briggs, 2010) suggesting that the skin-associated system of innate immunity may be of critical importance in host defense. Previous studies have described the purification and structural characterization of host defense peptides in skin secretions from *R. aurora* (Conlon et al., 2005), *R. boylii* (Conlon et al., 2003), *R. cascadae* (Conlon et al., 2007a), *R. draytonii* (Conlon et al., 2006), *R. luteiventris* (Conlon and Leprince, 2010; Goraya et al., 2000), and *R. muscosa* (Rollins-Smith et al., 2006). We now extend these studies to include characterization of the antimicrobial peptides in norepinephrine-stimulated skin secretions from specimens of the Oregon spotted frog *R. pretiosa* Baird and Girard, 1853. The animals had previously been infected experimentally with *B. dendrobatidis* but had proved resistant to developing chytridiomycosis (Padgett-Flohr and Hayes, in press).

R. pretiosa is a medium-sized (4.4–10.5 cm snout-to-vent length), highly aquatic frog that is closely associated with permanent water. Although once relatively widespread over an area from northeastern California to southwestern British Columbia, the species has disappeared from 70 to 90% of its geographical range (Pearl and Hayes, 2005) and is listed as vulnerable in the IUCN Red List of Threatened Species (Hammerson and Pearl, 2010). It is now restricted to isolated sites in western and south-central Washington and the east Cascades region of central and south-central Oregon. As well as chytridiomycosis, population declines have been caused by introduction of predators such as American bullfrogs (*Lithobates catesbeianus*) and non-native fish, habitat destruction, and pollution of groundwater by agrochemicals (Hayes and Jennings, 1986).

Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines (Conlon, 2008). Peptides from previously described families are given the suffix PR and isoforms are denoted by lower case letters e.g. brevinin-1PRa.

2. Experimental

2.1. Animals

All experiments with live animals were approved by the Animal Research Ethics Committee of UAE University (Protocol no. A21-09) and were carried out by authorized investigators under a program permit from the Washington Department of Fish and Wildlife. Full details of the procedures by which the animals were raised and infected with the chytrid fungus *B. dendrobatidis* are provided elsewhere (Padgett-Flohr and Hayes, in press). In brief, *R. pretiosa* eggs were collected from Conboy Lake National Wildlife Refuge, Klickitat County, Washington and reared in a chytrid-free environment at the Woodland Park Zoo (Seattle, Washington). Prior to experimentation, PCR analysis was used to verify that the animals tested negative for the pathogen. Full details of the PCR procedure are provided in Padgett-Flohr and Hayes (in press). In August 2009, recently metamorphosed animals ($n = 18$) were exposed to two *B. dendrobatidis* 284 strains: a cultured strain (JEL) provided by J.E. Longcore and a wild strain (FB) provided by F. Brem. PCR analysis demonstrated that all animals became infected by 11 days post-exposure but no animal died or exhibited signs of chyridiomycosis. After 90 days post-exposure, 15 out 18 animals had cleared their infection and were shown to be chytrid-free by PCR analysis.

2.2. Collection of skin secretions

Skin secretions were collected from chytrid-free, one year-old, sub-adult *R. pretiosa* ($n = 15$) in April, 2010. At the time of collection of secretions, the frogs were housed in a garden pool in the Mountain View area of Santa Clara, CA. Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body mass) and placed in a solution (100 ml) of distilled water for 15 min. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 ml) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions were pooled and separately passed at a flow rate of 2 ml/min through8 Sep-Pak C-18cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 ml).

2.3. Peptide purification

The pooled skin secretions, after partial purification on Sep-Pak cartridges, were injected onto a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm, and fractions (1 min) were collected. Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent (50 μl) in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Fractions associated with antimicrobial activity were successively chromatographed on a (1 cm × 25 cm) Vydac 214TP510 (C-4) column, a (1 cm × 25 cm) Vydac 208TP510 (C-8) column and a (1 cm × 25 cm) Vydac 219TP510 phenyl column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml/min.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2–4 kDa range. The accuracy of mass determinations was ±0.02%.

2.5. Antimicrobial and hemolytic activities

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD). Minimum inhibitory concentrations (MIC) of the purified peptides against reference strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25726), and *Candida albicans* (ATCC 90028) were measured by standard microdilution methods (Clinical Laboratory and Standards Institute, 2008a,b). In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of antibiotics (ampicillin for *S. aureus* and *E. coli*; amphotericin for *C. albicans*) as previously described (Conlon et al., 2007a).