ACADEMIC PRESS

Biochemical and Biophysical Research Communications 297 (2002) 361–367

BBRC

www.academicpress.com

Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*

Louise A. Rollins-Smith,[a,*] Laura K. Reinert,[a] Verma Miera,[b] and J.Michael Conlon[c]

[a] *Departments of Microbiology and Immunology and of Pediatrics, A-5301 Medical Center North, Vanderbilt University, Nashville, TN 37232, USA*
[b] *Department of Biology, Arizona State University, Tempe, AZ 85287, USA*
[c] *Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, 17666 Al Ain, United Arab Emirates*

Received 19 August 2002

Abstract

Populations of the Tarahumara frog *Rana tarahumarae* have decreased markedly in recent years in the northern part of their range. Infection by the chytrid fungus *Batrachochytrium dendrobatidis* has been implicated in these declines. To determine whether antimicrobial peptides in the skin provide protection against this pathogen, norepinephrine-stimulated skin secretions were tested for their ability to inhibit growth of *B. dendrobatidis* in vitro. After concentration, crude mixtures of skin peptides inhibited the growth of the chytrid in a concentration-dependent manner. Proteomic analysis led to the identification and characterization of three peptides belonging to the brevinin-1 family of antimicrobial peptides and three belonging to the ranatuerin-2 family. The two most abundant peptides, ranatuerin-2TRa (GIMDSIKGAAKEIAGHLLDNLKCKITGC) and brevinin-1TRa (FLPVIAGIAANVLPKLFCKLTKRC), were active against *B. dendrobatidis* (MIC of 50 μM for ranatuerin-2TRa and 12.5 μM for brevinin-1TRa against zoospores). These data clearly show that antimicrobial peptides in the skin secretions of the Tarahumara frog are active against *B. dendrobatidis* and should provide some protection against infection. Therefore, the observed susceptibility of these frogs to this pathogen in the wild may be due to the effects of additional environmental factors that impair this innate defense mechanism, leading to the observed population declines. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Antimicrobial peptide; *Batrachochytrium dendrobatidis*; Brevinin; Chytrid; Frog skin; *Rana tarahumarae*; Ranatuerin

Although mostly a Mexican species, the historical range of Tarahumara frogs (*Rana tarahumarae*) once extended northward to a few isolated populations in the Madrean Sky Islands of extreme southern Arizona [1]. Although these Arizona populations were isolated, Tarahumara frogs were locally abundant in their preferred habitat of deep perennial plunge pools in bedrock canyons [2]. This changed dramatically beginning in the mid-1970s. Populations in the Atascosa–Pajarito–Tumacacori mountain complex near Nogales and in the Santa Rita Mountains southeast of Tucson declined severely by the late 1970s. In 1983, the last confirmed report of a Tarahumara frog in Arizona was a dead female found in Big Casa Blanca Canyon of the Santa Ritas [3,4]. The extirpation of the Tarahumara frog from Arizona, coupled with the decline of other Arizona native ranids, including the sympatric Chiricahua leopard frog (*Rana chiricahuensis*) and the lowland leopard frog (*Rana yavapaiensis*), is a matter of concern for both amphibian researchers and government agencies that monitor and manage wildlife populations. Cadmium deposited from smelting operations was initially proposed as a possible cause of declines [3]. Heavy metal toxicity can disturb water and ion balance and, in combination with stress, can cause electrolyte imbalances severe enough to induce seizures, neuromuscular dysfunction, and death. These symptoms matched those of ill animals collected in the field. No other convincing theory for the decline of Tarahumara frogs emerged until the recent linkage of amphibian declines in Australia and Central America with chytrid fungus infections ([5–7], reviewed in [8,9]). The physical symptoms described by Hale and Jarchow in 1988 are also con-

* Corresponding author. Fax: 1-615-343-8648.
E-mail address: louise.rollins-smith@vanderbilt.edu (L.A. Rollins-Smith).

PII: S0006-291X(02)02217-9

sistent with chytridiomycosis [9]. Hale and the Tarahumara Frog Conservation Team [10,11] reexamined specimens from dead or moribund individuals collected in 1982 and 1985 from several sites in Sonora, Mexico. These specimens all showed chytrid infections; however, other apparently healthy individuals with mild chytrid infections were also found. Thus, *B. dendrobatidis* is likely to be involved in recent declines of the Tarahumara frogs, but additional factors may determine survival.

B. dendrobatidis infects and replicates in the keratinized epithelium of the skin. Fungal cells encyst, enlarge, form a zoosporangium, and fragment to produce flagellated zoospores. The zoospores are released from discharge papillae and may infect other areas of the skin of the same host or may infect a new host [5–7]. The initial infection involves only the keratinized epithelium and local cell-mediated immune responses do not seem to develop [7].

It has been proposed that the synthesis of polypeptides with a broad spectrum of antimicrobial activity in the granular glands present in the skin of anurans (frogs and toads) is an important feature of the defense strategy of these vertebrates (reviewed in [12–14]). Skin secretions generally contain multiple antimicrobial peptides with distinct spectra of activity, and it has been suggested that this molecular diversity is important in protecting the animal from invasion by a wide array of different microorganisms [15]. The peptides range in size from 12 to 46 amino acid residues, and while there is no consensus amino acid sequence that defines antimicrobial activity, they are generally cationic, hydrophobic, and adopt an amphipathic α-helical conformation upon binding to the cell membrane (reviewed in [12–14,16]). Their precise mechanism of action remains unclear, but the antimicrobial peptides are believed to bind to charged residues on target cells and produce cell death by disruption of the cell membrane into peptide-coated vesicles in a manner similar to that of a detergent (reviewed in [14,17–19]).

Numerous studies (reviewed in [12,13]) have demonstrated that peptides isolated from frog skin are active, with varying degrees of potency, against a wide range of laboratory strains of Gram-negative and Gram-positive bacteria and against certain yeasts. Previously, we have shown that they are also active against several amphibian pathogens including *B. dendrobatidis* [20–22]. Here, we show that such peptides isolated from the Tarahumara frog can inhibit growth of the pathogenic chytrid in vitro, and thus, they would seem to be capable of providing the organism with protection against infection. This suggests that environmental factors, such as toxic chemicals or cold temperatures, may inhibit this innate defense mechanism, allowing a mild controlled infection to become lethal.

Materials and methods

Animals. *Rana tarahumarae* were collected as an egg mass in Mexico by S. Hale. J. Rorabaugh of the US Fish and Wildlife Service raised the eggs to tadpoles and transferred some to Arizona State University. These animals were raised to maturity at Arizona State University and two frogs were transferred to the Rollins-Smith laboratory for peptide collection.

Collection of skin secretions. A single individual of *R. tarahumarae* (23.3 g) was injected bilaterally with 2 nmoles/gram body weight of [±]-norepinephrine hydrochloride in a volume of 0.23 ml via the dorsal lymph sac. Immediately following injection, the frog was placed in 100 ml collection buffer (25 mM NaCl and 25 mM ammonium acetate, pH 7.0) [23] and skin secretions were allowed to accumulate in the buffer for a period of 15 min. Following release of skin secretions, the frog was removed and the buffer was acidified by addition of 1.0 ml trifluoroacetic acid (TFA). The skin secretions were partially purified by passage over a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA) as previously described [24,25]. Protein concentration was determined using the Micro BCA Assay (Pierce, Rockford, IL) following manufacturer's instructions, except that bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve [26]. A sample of the crude peptides was adjusted to pH 6.6 and tested for anti-chytrid growth inhibitory activity. Following a rest interval of 21 days, skin secretions were collected a second time following the same procedure. The acidified skin secretions and the partially purified material were transferred to the Conlon laboratory for further purification of the peptides.

Purification of the antimicrobial peptides. After partial purification on Sep-Pak cartridges as previously described [24,25], the skin secretions from the two stimulations were pooled, centrifuged (12,000*g* for 3 min) and injected onto a (25 × 1 cm) Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) TFA/water. 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 at a flow rate of 2 ml/min. Absorbance was monitored at 214 nm and fractions (1 min) were collected. Aliquots (10 μl) of fractions with retention times between 48 and 62 min were analyzed by electrospray mass spectrometry using a Perkin–Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was 0.02%. Individual peptides were purified to near homogeneity (as assessed by peak symmetry and mass spectrometry) by sequential rechromatography on (25 × 1 cm) Vydac 214TP510 (C-4) and (25 × 1 cm) Vydac 219TP510 (phenyl) columns. The concentration of acetonitrile in the eluting solvent was raised from 24% to 56% over 40 min and the flow rate was 2 ml/min.

Structural characterization. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 491 Procise sequenator. Amino acid compositions were determined by precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate using a Waters AccQ Tag system with fluorescence detection and separation of the amino acid derivatives by reversed-phase HPLC. Hydrolysis in 5.7 M HCl (24 h at 110 °C) of approximately 1 nmol peptide was carried out.

Growth inhibition assay. *B. dendrobatidis* (isolate 197) [6] was obtained from Joyce Longcore and maintained in culture as described previously [21,22]. Mature cells or zoospores were plated with or without peptides as described previously [21,22]. Briefly, 5×10^4 mature cells or 5×10^5 zoospores in a volume of 50 μl were plated in replicates of five in a 96-well flat-bottomed microtiter plate (Costar 3596, Corning, Corning, NY, USA) with or without addition of 50 μl serial dilutions of peptides in broth. The plates were covered, wrapped in plastic wrap to limit moisture loss, and incubated on a laboratory bench at 23 °C. To determine maximal growth (positive control for growth), some wells received 50 μl broth without peptide. To determine the value for maximal inhibition (negative control for growth), some

wells (on a separate plate) received 50 μl broth containing 0.4% paraformaldehyde (PF) [21,22]. Growth at 1–5 days (23 °C) was measured as increased optical density at 492 nm (OD_{492}) with an ELISA plate reader. For growth inhibition assays using zoospores, the incubation period was extended up to seven days. Minimal inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was detectable. That is, the OD_{492} was not significantly greater than that observed for negative control wells containing PF.

Results

Purification of the peptides

The elution profile on a Vydac C-18 semipreparative column of the combined skin secretions and washings, after partial purification on Sep-Pak cartridges, is shown in Fig. 1. Analysis by electrospray mass spectrometry demonstrated that the fractions denoted by zone A contained a major component of mass 2898 (subsequently shown to be ranatuerin-2TRa). The fractions denoted by zone B contained a major component of mass 2614 (subsequently shown to be brevinin-1TRa). The fractions denoted by zone C were heterogeneous and contained peptides with masses 2884 (ranatuerin-2TRb), 2950 (ranatuerin-2TRc), 2591 (brevinin-1TRb), and 2581 (brevinin-1TRc). Ranatuerin-2TRa (Fig. 2A) and brevinin-1TRa (Fig. 2B) were purified to near homogeneity by rechromatography on a Vydac C-4 semipreparative column. As shown in Fig. 2C, partial separation of ranatuerin-2TRb, ranatuerin-2TRc, brevinin-1TRb, and brevinin-1TRc was achieved on the Vydac C-4 column and the peptides were purified to apparent homogeneity by a final chromatography on a semipreparative Vydac phenyl column. The final yields of the purified peptides were: ranatuerin-2TRa 138 nmol, ranatuerin-2TRb 25 nmol, ranatuerin-2TRc 9 nmol, brevinin-1TRa 110 nmol, brevinin-1TRb 27 nmol, and brevinin-1TRc 13 nmol.

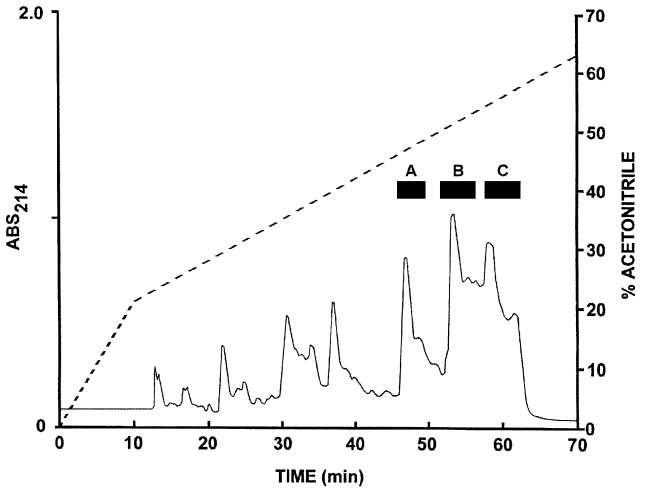

Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of the skin secretions of the Tarahumara frog, after partial purification on Sep-Pak cartridges. The dashed line shows the concentration of acetonitrile in the eluting solvent. The fractions denoted by bar A contained ranatuerin-2TRa, by bar B contained brevinin-1TRa, and by bar C contained ranatuerin-2TRb, ranatuerin-2TRc, brevinin-1TRb, and brevinin-1TRc.

Structural characterization

The primary structures of the purified peptides were determined without ambiguity by automated Edman degradation and their amino acid sequences are shown in Fig. 3. In all cases, the proposed amino acid sequences were confirmed by amino acid composition analysis and electrospray mass spectrometry. The presence of a cysteine bridge in all peptides was also confirmed by electrospray mass spectrometry.

Activity against B. dendrobatidis

From a single NE-induction of a single frog, approximately 5.8 mg peptides were recovered after Sep-Pak concentration. The mixture of peptides inhibited growth at all concentrations above 29 μg/ml and minimal inhibitory concentration (MIC) was about 57 μg/ml (Fig. 4). Purified brevinin-1TRa inhibited growth of mature chytrids at concentrations above 12.5 μM with an MIC of 100 μM (Fig. 5A) and ranatuerin-2TRa inhibited growth of mature cells at concentrations above 50 μM (Fig. 5B). Both peptides were more effective against purified zoospores. Brevinin-1TRa inhibited growth of zoospores at concentrations above 6.25 μM with an MIC of 12.5 μM (Fig. 5C) and ranatuerin-2TRa inhibited growth of zoospores at concentrations above 12.5 μM with an MIC of 50 μM (Fig. 5D).

Discussion

The present study examined the activity of antimicrobial peptides from the Tarahumara frog against the chytrid fungus that has been associated with population declines in Australia, Central America, the Western US, and Europe [5–7,27]. Our work illustrates the power of reversed-phase HPLC coupled with electrospray mass spectrometry for the separation and identification of peptide components in a complex mixture. The application of these techniques to analysis of the skin secretions of the Tarahumara frog led to the characterization of six new peptides belonging to the previously described ranatuerin-2 and brevinin-1 families, first isolated from extracts of the skins of *R. catesbeiana* [24] and *Rana brevipoda porsa* [28], respectively.

Frogs from the genus *Rana* constitute a diverse group with an estimated 250 species worldwide, distributed in all continents, except Antarctica [29]. At this time, skin secretions and/or skin extracts have been analyzed for 10

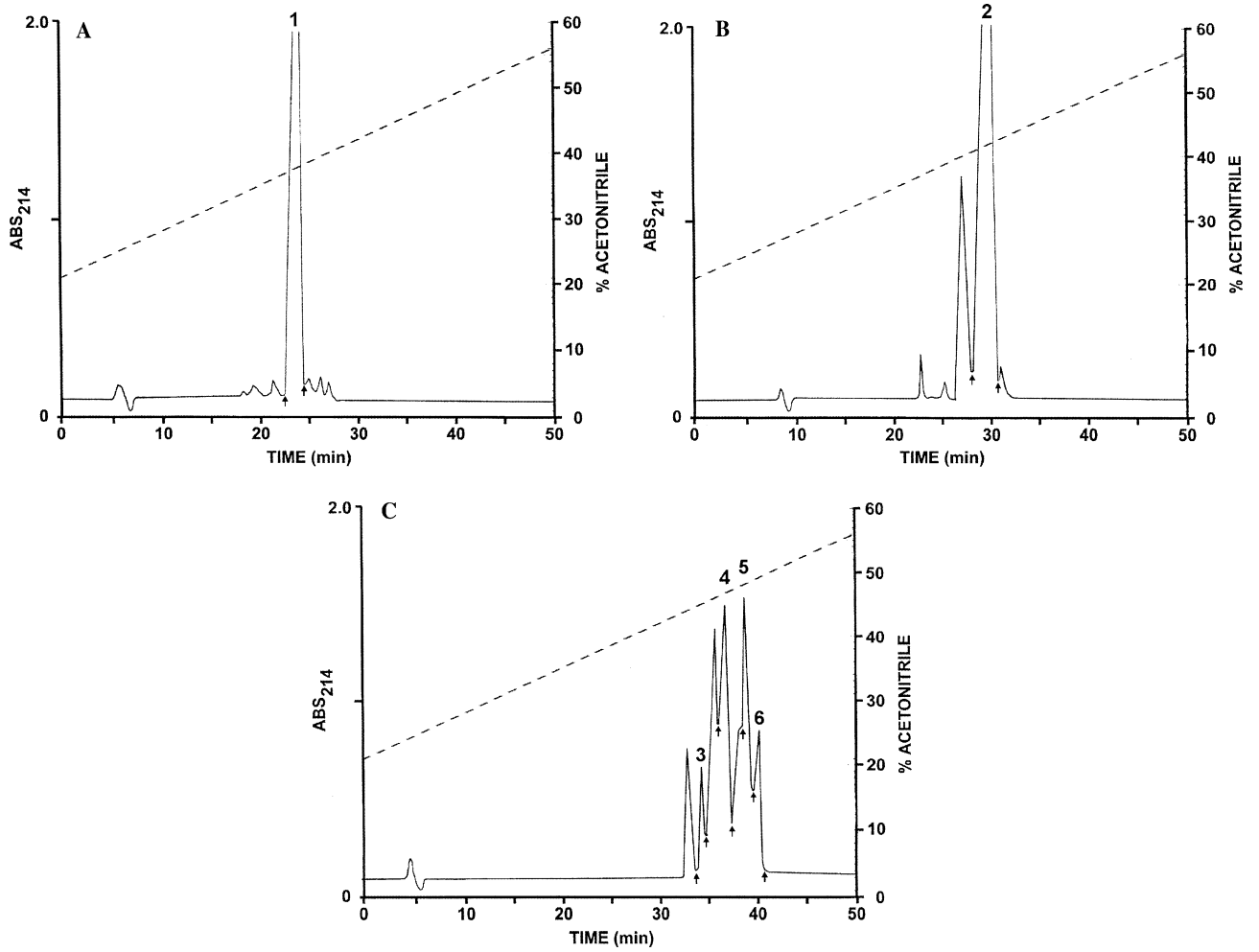

Fig. 2. Purification on a semipreparative Vydac C-4 column of (A) ranatuerin- 2TRa (peak 1), (B) brevinin-1TRa (peak 2), and (C) ranatuerin-2TRc (peak 3), ranatuerin-2TRb (peak 4), brevinin-1TRb (peak 5), and brevinin-1TRc (peak 6). The arrows show where peak collection began and ended.

Brevinin-1TRa FLPVIAGIAANVLPKLFCKLTKRC

Brevinin-1TRb FLPFIASMAAKLVPKLVCAITKKC

Brevinin-1TRc FLPVLAGIAANVLPTLICKLTRRC

Brevinin-1 FLPVLAGIAAKVVPALFCKITKKC

Ranatuerin-2TRa GIMDSIKGAAKEIAGHLLDNLKCKITGC

Ranatuerin-2TRb GILDTLKNVAKNVAAGLLDNIKCKITGC

Ranatuerin-2TRc GIFDTIKNVAKNMAAGLLDNIKCKITGC

Ranatuerin-2 GLFLDTLKGAAKDVAGKLEGLKCKITGCKLP

Fig. 3. A comparison of the primary structures of the peptides isolated from *R. tarahumarae* with ranatuerin-2 first isolated from the skin of the American bullfrog *R. catesbeiana* and brevinin-1 first isolated from the skin of the Asian frog *R. brevipoda porsa*. Only those amino acids that are shaded have been conserved among the peptides of each family.

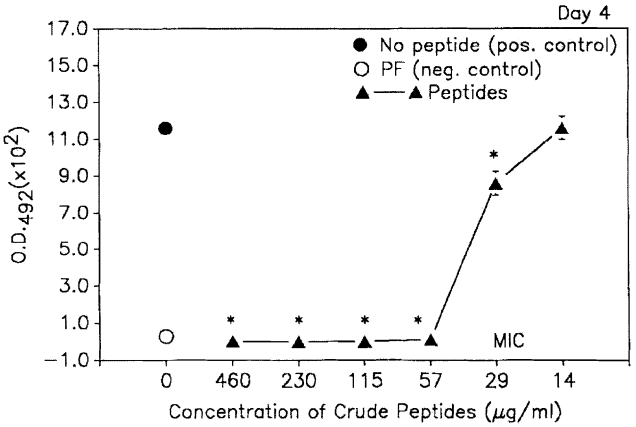

Fig. 4. Growth inhibition of *B. dendrobatidis* mature cells at 4 days of culture by skin secretions of *R. tarahumarae* partially purified and concentrated by passage over a C-18 Sep-Pak cartridge. Each data point represents the means ± standard error (SE) of five or more replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Significantly less growth than positive controls (one-tailed Student's *t* test, $p \leqslant 0.005$). MIC is the lowest concentration at which no growth was detected.

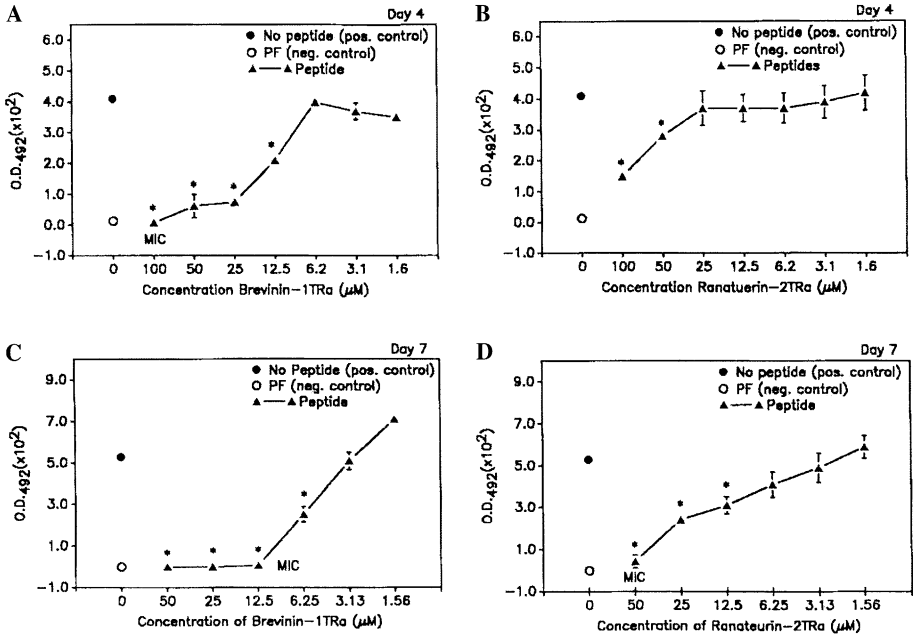

Fig. 5. Growth inhibition of *B. dendrobatidis* mature cells at 4 days of culture by (A) brevinin-1TRa and (B) ranatuerin-2TRa. Growth inhibition of *B. dendrobatidis* zoospores at 7 days of culture by (C) brevinin-1TRa and (D) ranatuerin-2TRa. Each data point represents the means ± SE of five or more replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Significantly less growth than positive controls (one-tailed Student's t test, $p \leqslant 0.005$). MIC is the lowest concentration at which no growth was detected.

species of North American frogs of the genus *Rana* [24,25,30–35]. As shown in Fig. 6, peptides belonging to 10 different families (brevinin-1, ranalexin, ranatuerin-1, ranatuerin-2, esulentin-1, esculentin-2, temporin, palustrin-1, palustrin-2, and palustrin-3) have been identified and the distribution and multiplicity of the molecular forms are quite species-specific. The degree of diversity ranges from a single peptide in the case of the wood frog, *R. sylvatica* [30] to 22 peptides in the case of the pickerel frog, *R. palustris* [31]. Peptides of the brevinin-1 and ranatuerin-2 families appear to have the widest distribution in those species of North American ranids examined to date. It is probable that the multiple members of each peptide family have arisen from successive duplications of an ancestral gene. If this hypothesis is correct, the data in Fig. 3 demonstrate that the peptides of the brevinin-1 and ranatuerin-2 families have undergone a very rapid rate of evolution. Only 9 out of 24 amino acid residues in the brevinin-1 peptides and 10 out of 28 residues in the ranatuerin-2 peptides have been conserved but the cationicity and potential to form an α-helix [36] have been preserved.

Previous studies [24,25,30,31,33] have shown that peptides of the brevinin-1 family isolated from the skins of North American frogs show broad spectrum antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus*, Gram-negative bacteria such as *Escherichia coli*, and against the yeast *Candida albicans*. MIC values against the three microorganisms are generally in the range 1–20 μM. Peptides belonging to the ranatuerin-2 family have low antimicrobial potencies against *S. aureus* and *C. albicans* (MIC >100 μM) but are more active against *E. coli* (MIC in the range 2–50 μM) [24,25,34,35]. Recent work has also shown that ranatuerin-2P, originally isolated from *R. pipiens* skin [25], will also inactivate frog virus 3, a potentially pathogenic virus infecting anurans [20].

Prior to this study, we hypothesized that species that are susceptible to chytridiomycosis might have relatively poor repertoires of antimicrobial peptides effective against *B. dendrobatidis*. However, it is clear that *R. tarahumarae*, a species that has declined in the presence of chytridiomycosis within susceptible populations, has a set of skin peptides that are highly effective as a

		Reference
R. tarahumarae	Brevinin-1 (3), Ranatuerin-2 (3)	This study
R. catesbeiana	Brevinin-1 (1), Ranalexin (1) Ranatuerin-1(1), Ranatuerin-2 (3), Temporin (5)	[24]
R. grylio	Ranatuerin-1 (2), Ranatuerin-2 (1), Ranalexin (1), Temporin (4)	[35]
R. clamitans	Ranatuerin-1 (1), Ranatuerin-2 (2), Ranalexin (2), Temporin (5)	[34]
R. luteiventris	Brevinin-1 (1), Esculentin-2 (1), Ranatuerin-2 (2), Temporin (3)	[25]
R. pipiens	Brevinin-1 (4), Esculentin-2 (1), Ranatuerin-2 (1), Temporin (1)	[25]
R. berlandieri	Brevinin-1 (6), Esculentin-2 (1), Ranatuerin-2 (1)	[25]
R. sphenocephala	Brevinin-1 (3)	[33]
R. sylvatica	Brevinin-1(1)	[30]
R. palustris	Brevinin-1 (3), Esculentin-1 (2), Esculentin-2 (1), Ranatuerin-2 (6) Temporin (1), Palustrin-1 (4), Palustrin-2 (3), Palustrin-3 (2)	[31]

Fig. 6. Distribution of the molecular forms of the antimicrobial peptides isolated from the skins of North American ranid frogs. The value in parentheses is the number of peptides belonging to a particular family that are synthesized in that species.

crude mixture at a concentration as low as about 57 μg/ml (Fig. 4). Two of the purified skin peptides were effective against zoospores at 12.5 μM (brevinin-1TRa) and 50 μM (ranatuerin-2TRa) (Fig. 5). Thus, it would seem that *R. tarahumarae* has a good antimicrobial peptide defense system in place. Why then do Tarahumara frogs appear to be highly susceptible to chytridiomycosis?

There are a number of possible explanations for the apparent susceptibility to chytridiomycosis in the presence of active skin peptides. First, it is unclear in any species how much of each peptide is present on the skin of a resting frog. In the presence of agents that stimulate the sympathetic nervous system, large amounts can be secreted. As much as 50 mg can be collected from a single individual [37, and M. Zasloff, unpublished observation]. From one individual of *R. tarahumarae* and using a single relatively mild norepinephrine stimulation, we collected approximately 5.8 mg peptides for this study. However, resting frogs may have a much more limited amount of antimicrobial peptides available to prevent infection by a swimming zoospore. Peptides that are secreted and would be present in a terrestrial environment may be rapidly washed away in an aquatic environment.

A second possible explanation is that under normal conditions, antimicrobial peptide defenses do limit mild infections, but external factors may inhibit production or release. Possible factors that may interfere with antimicrobial peptide production are stress that elevates glucocorticoids [38] and cold temperatures [30]. Other factors that have not yet been studied for their effects on antimicrobial peptide production and release are pesticides, ultraviolet irradiation, and heavy metals. Each of these factors has been linked to amphibian declines [3,39–41]. Hale et al. [12] suggested that environmental stressors, such as cold winters or heavy metal intoxication, may have exacerbated the effects of normally mild chytrid infections, leading to the extirpation of the most northern populations of Tarahumara frogs. To successfully return this species to Arizona, it is clear that further work is needed to understand the possible involvement of environmental factors and chytridiomycosis in Tarahumara frog declines in Arizona and northern Sonora.

Acknowledgments

We thank Dr. Donald Babin, Creighton University, for amino acid analyses and Ms. Eva Lovas, Creighton University, for mass spectrometry measurements. Collection of *R. tarahumarae* eggs was supported by a grant to S. Hale from the US Fish and Wildlife Service. The current work was supported by a grant from Restoragen Inc., Lincoln, Nebraska (to J.M.C.), and by an NSF IRCEB Grant IBN- 9977063 (James Collins, P.I.) and NSF Grant IBN-0131184 (to L.R.-S.).

References

[1] S.F. Hale, F. Retes, T.R. Van Devender, New populations of *Rana tarahumarae* (Tarahumara frog) in Arizona, J. Arizona Acad. Sci. 11 (1977) 134–135.
[2] S.F. Hale, C.J. May, Status report for *Rana tarahumarae* Boulenger. Arizona Natural Heritage Program, Tucson. Report to Office of Endangered Species, US Fish and Wildlife Service, Albuquerque, New Mexico, 1983.
[3] S.F. Hale, J.L. Jarchow, Status of the Tarahumara Frog *Rana tarahumarae* in the United States and Mexico: Part II. Report to Arizona Game and Fish Department and Office of Endangered Species, US Fish and Wildlife Service, Albuquerque, New Mexico, 1988.
[4] S.F. Hale, A survey of historical and potential habitat for the Tarahumara Frog (*Rana tarahumarae*) in Arizona. Report to Arizona Game and Fish Department and US Forest Service, Coronado Nation Forest, 1992.
[5] L. Berger, R. Speare, P. Daszak, D.E. Green, A.A. Cunningham, C.L. Goggin, R. Slocombe, M.A. Ragan, A.D. Hyatt, K.R. McDonald, H.B. Hines, K.R. Lips, G. Marantelli, H. Parkes, Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America, Proc. Natl. Acad. Sci. USA 95 (1998) 9031–9036.
[6] J.E. Longcore, A.P. Pessier, D.K. Nichols, *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians, Mycologia 91 (1999) 219–227.
[7] A.P. Pessier, D.K. Nichols, J.E. Longcore, M.S. Fuller, Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp. and White's tree frogs (*Litorea caerulea*), J. Vet. Diagn. Invest. 11 (1999) 194–199.
[8] C. Carey, N. Cohen, L. Rollins-Smith, Amphibian declines: an immunological perspective, Dev. Comp. Immunol. 23 (1999) 459–472.
[9] P. Daszak, L. Berger, A.A. Cunningham, A.D. Hyatt, D.E. Green, R. Speare, Emerging infectious diseases and amphibian population declines, Emerg. Infect. Dis. 5 (1999) 735–748.
[10] S. F. Hale, The Status of the Tarahumara Frog in Sonora Mexico. Report to United States Fish and Wildlife Service, Phoenix, AZ, 2001.
[11] S.F. Hale, P.C. Rosen, J.L. Jarchow, G.A. Bradley. 2002. Effects of the Chytrid Fungus on the Tarahumara Frog in Sonora Mexico. Abstract in Current Research on Herpetofauna of Sonoran Desert II, Tucson, AZ.
[12] P. Nicolas, A. Mor, Peptides as weapons against microorganisms in the chemical defense system of vertebrates, Annu. Rev. Microbiol. 49 (1995) 277–304.
[13] M. Simmaco, G. Mignogna, D. Barra, Antimicrobial peptides from amphibian skin: What do they tell us?, Biopolym. Peptide Sci. 47 (1998) 435–450.
[14] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[15] A. Mor, K. Hani, P. Nicolas, The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms, J. Biol. Chem. 269 (1994) 31635–31641.
[16] A. Tossi, L. Sandri, A. Giangaspero, Amphipathic, α-helical antimicrobial peptides, Biopolymers 55 (2000) 4–30.
[17] K. Matsuzaki, Why and how are peptide–lipid interactions utilized for self defense? Magainins and tachyplesins as archetypes, Biochim. Biophys. Acta 1462 (1999) 1–10.
[18] Y. Shai, Mechanism of the binding, insertion, stabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim. Biophys. Acta 1462 (1999) 55–70.

[19] L. Yang, T.M. Weiss, R.I. Lehrer, H.W. Huang, Crystallization of antimicrobial pores in membranes: magainin and protegrin, Biophys. J. 79 (2000) 2002–2009.
[20] V.G. Chinchar, J. Wang, G. Murti, C. Carey, L. Rollins-Smith, Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin, Virology 288 (2001) 351–357.
[21] L.A. Rollins-Smith, J.K. Doersam, J.E. Longcore, S.K. Taylor, J.C. Shamblin, C. Carey, M.A. Zasloff, Antimicrobial peptide defenses against pathogens associated with global amphibian declines, Dev. Comp. Immunol. 26 (2002) 63–72.
[22] L.A. Rollins-Smith, C. Carey, J. Longcore, J.K. Doersam, A. Boutte, J.E. Bruzgal, J.M. Conlon, Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines, Dev. Comp. Immunol. 26 (2002) 471–479.
[23] M.G. Giovannini, L. Poulter, B.W. Gibson, D.H. Williams, Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones, Biochem. J. 243 (1987) 113–120.
[24] J. Goraya, F.C. Knoop, J.M. Conlon, Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, *Rana catesbeiana*, Biochem. Biophys. Res. Commun. 250 (1998) 589–592.
[25] J. Goraya, Y. Wang, Z. Li, M. O'Flaherty, F.C. Knoop, J.E. Platz, J.M. Conlon, Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs, *Rana luteiventris*, *Rana berlandieri*, and *Rana pipiens*, Eur. J. Biochem. 267 (2000) 894–900.
[26] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[27] J. Bosch, I. Martínez-Solano, M. Garca-Pars, Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain, Biol. Conserv. 97 (2001) 331–337.
[28] N. Morikawa, K. Hagiwara, T. Nakajima, Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*, Biochem. Biophys. Res. Commun. 189 (1992) 184–190.
[29] W.E. Duellman, L. Trueb, Biology of Amphibians, The Johns Hopkins University Press, Baltimore and London, 1994.
[30] B. Matutte, K.B. Storey, F.C. Knoop, J.M. Conlon, Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica* in response to environmental stimuli, FEBS Lett. 483 (2000) 135–138.
[31] Y.J. Basir, F.C. Knoop, J. Dulka, J.M. Conlon, Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin *N* isolated from the skin secretions of the North American pickerel frog, *Rana palustris*, Biochim. Biophys. Acta 1543 (2000) 95–105.
[32] D.P. Clark, S. Durell, W.L. Maloy, M. Zasloff, Ranalexin A novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin, J. Biol. Chem. 269 (1994) 10849–10855.
[33] J.M. Conlon, T. Halverson, J. Dulka, J.E. Platz, F.C. Knoop, Peptides with antimicrobial activity of the brevinin-1 family isolated from skin secretions of the Southern leopard frog, *Rana sphenocephala*, J. Peptide Res. 54 (1999) 522–527.
[34] T. Halverson, Y.J. Basir, F.C. Knoop, J.M. Conlon, Purification and characterization of antimicrobial peptides from the skin of the North American green frog *Rana clamitans*, Peptides 21 (2000) 469–476.
[35] J.B. Kim, T. Halverson, Y.J. Basir, J. Dulka, F.C. Knoop, J.M. Conlon, Purification and characterization of antimicrobial peptides from skin extracts and skin secretions of the North American pig frog *Rana grylio*, Regul. Peptides 90 (2000) 53–60.
[36] J. Garnier, D. Osguthorpe, B. Robson, Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins, J. Mol. Biol. 120 (1978) 97–121.
[37] M.J. Tyler, D.J.M. Stone, J.H. Bowie, A novel method for the release and collection of dermal, glandular secretions from the skin of frogs, J. Pharmacol. Toxicol. Meth. 28 (1992) 199–200.
[38] M. Simmaco, A. Boman, M.L. Mangoni, G. Mignogna, R. Miele, D. Barra, H.G. Boman, Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin, FEBS Lett. 416 (1997) 273–275.
[39] D.W. Sparling, G.M. Fellers, L.L. McConnell, Pesticides and amphibian population declines in California, USA, Environ. Toxicol. Chem. 20 (2001) 1591–1595.
[40] E.M. Middleton, J.R. Herman, E.A. Celarier, J.W. Wilkinson, C. Carey, R.J. Rusin, Evaluating ultraviolet radiation exposures determined from satellite data at sites of amphibian declines in Central and South America, Conserv. Biol. 15 (2001) 914–929.
[41] A.R. Blaustein, L.K. Belden, A.C. Hatch, L.B. Kats, P.D. Hoffman, J.B. Hays, A. Marco, D.P. Chivers, J.M. Kiesecker, Ultraviolet radiation and amphibians, in: C.S. Cockell, A.R. Blaustein (Eds.), Ecosystems, Evolution, and Ultraviolet Radiation, Springer, New York, 2001, pp. 63–79.