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Antimicrobial peptide defenses against pathogens associated with global amphibian declines

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Abstract

Global declines of amphibian populations are a source of great concern. Several pathogens that can infect the skin have been implicated in the declines. The pathogen most frequently associated with recent die-offs is a chytrid fungus, *Batrachochytrium dendrobatidis*. A second fungus, *Basidiobolus ranarum,* was isolated from declining populations of Wyoming toads. A third pathogen, *Aeromonas hydrophila*, is an opportunistic bacterium found in healthy frogs, but capable of inducing disease. Among the immune defense mechanisms used by amphibians is the production of antimicrobial peptides in granular glands in the skin. These packets of natural antibiotics can be emptied onto the skin when the amphibian is injured. To determine whether antimicrobial skin peptides defend against these amphibian pathogens, six peptides (magainin I, magainin II, PGLa, CPF, ranalexin, and dermaseptin), from three species, and representing three structurally different families of peptides, were tested in growth inhibition assays. We show here that the peptides can kill or inhibit growth of both fungi but not *Aeromonas.* Although each peptide varied in its effectiveness, at least one from each species was effective against both fungi at a concentration of about 10–20 μM. This is the first direct evidence that antimicrobial peptides in the skin can operate as a first line of defense against the organisms associated with global amphibian declines. It suggests that this innate defense mechanism may play a role in preventing or limiting infection by these organisms.

Keywords: Amphibians; Amphibian declines; Antimicrobial peptides; *Batrachochytrium*; Chytrid; Frog; Magainin; Ranalexin

1. Introduction

Amphibian populations have been declining in many parts of the world since the 1960s (reviewed in Refs. [1–3]). The mass mortalities were first documented in the 1970s with toads and frogs in the western US [4–9], southern Canada [10,11], and Brazil [12,13]. Mass mortalities in Central America and Australia have been described more recently. The die-offs in these areas, and in the US, are continuing [14–19].

Many possible causes for amphibian population declines have been proposed. A review of the patterns of declines suggests that, along with man-made changes like habitat destruction and introduction of

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xenobiotics, one or more emerging diseases may be responsible for recent declines [1,2]. Much effort has gone into a search for possible pathogens that could be the cause of mortality in these populations. Four pathogens have been identified that appear to meet the strict criteria set by microbiologists to establish a microbial pathogen as the cause of a disease (reviewed in Ref. [1]). The pathogen most frequently associated with recent amphibian die-offs is a newly described chytrid fungus, *Batrachochytrium dendrobatidis*, named for the blue poison dart frog (*Dendrobates auratus*) from which it was isolated [17,20,21]. A second fungus was isolated from clinically ill individuals from declining populations of Wyoming toads (*Bufo baxteri*) and was identified as *Basidiobolus ranarum* [22,23]. This fungus is a common inhabitant in the gut of amphibians [24–26], and it has been reported to be pathogenic for humans [27–29]. Mass die-offs of tiger salamanders (*Ambystoma tigrinum*) in Arizona and the common frog (*Rana temporaria*) in England appear to be caused by a third kind of pathogen. The pathogen causing these die-offs is characterized as an iridovirus based on morphology and host pathology [30,31]. A fourth pathogen associated with amphibian declines is the bacterium *Aeromonas hydrophila*. It appears to be an opportunist found on the skin and in the digestive tracts of healthy frogs [32], but capable of inducing disease in South African clawed frogs (*Xenopus laevis*) [33] and American toads *(Bufo americanus*) [34], especially when the animals are stressed [35,36].

All of the organisms described above can infect the skin. The chytrid fungus colonizes keratinized epithelium [17,20,21]. *Basidiobolus* can be transmitted to healthy toads by direct exposure of abraded skin [23]. The skin, as well as liver, can be colonized by the iridoviruses [30,31]. *A. hydrophila* is found on the skin and in the digestive tracts [32,33]. Thus, the skin as a defensive barrier seems to be central to protection from each of these pathogens. Little is known about immune defense mechanisms in amphibian skin. However, one defense mechanism that has generated interest is the production of antimicrobial peptides by specialized granular glands in the skin of many amphibians (reviewed in Refs. [37,38]). A growing number of these antimicrobial peptides, with sizes ranging from 10 to 46 amino acid residues, have been described. Each species appears to produce its own unique set of peptides with activity against a variety of organisms [39].

To understand the possible involvement of antimicrobial peptides produced by the granular glands in preventing or limiting infection by skin pathogens, we tested the activity of six peptides derived from three species. The peptides tested were magainin I, magainin II [40], PGLa (peptide with amino terminal glycine and carboxyl terminal leucinamide) [41], CPF (caerulein precursor fragment) [42], ranalexin [43], and dermaseptin [44]. The magainins, PGLa, and CPF were originally isolated from *X. laevis* and form linear α-helices that have wide spectrum antimicrobial activity [37–42]. Ranalexin was identified in the skin of the bullfrog (*Rana catesbeiana*) [43]. It differs from the others because of two cysteine residues at positions 1 and 7, when counting from the carboxyl terminus. This peptide is homologous to the brevinins from the skin of *Rana brevipoda porsa* [45] and other species of *Rana* [46], the pipinins isolated from *Rana pipiens* [47], and the esculentins isolated from *Rana esculenta* [48,49]. Dermaseptin comes from the skin of *Phyllomedusa sauvagii*. Like the magainins, it forms a linear α-helix and has broad-spectrum antimicrobial activity [37,44]. Although these peptides have been tested against mammalian pathogens in a search for novel antimicrobials, they have not previously been tested against amphibian pathogens.

We show here that all of the peptides tested can inhibit growth of *B. dendrobatidis* and *B. ranarum* but not *A. hydrophila*. This study is the first demonstration that skin-derived antimicrobial peptides can deter the growth of the specific pathogens associated with amphibian declines and suggests that this innate defense mechanism may play a role in preventing or limiting infection.

2. Materials and methods

2.1. Culture and maintenance of pathogens

B. dendrobatidis was isolated from a diseased blue poison dart frog (*D. auratus*) by J.E.L. [20]. It was grown on TGhL agar (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, and 10 g agar per 1 l of glass distilled water) or in H broth (10 g tryptone

and 3.2 g glucose per 1 l of glass distilled water) at 22–23°C. Broth cultures were passaged twice weekly to assure that cells were in an active phase of growth. *B. ranarum* was isolated from diseased Wyoming toads by S.K.T. [22,23]. It was grown on Sabouraud dextrose agar (Difco 0109-17) (Difco, Detroit, MI, USA) at 23°C. For growth inhibition assays in broth, it was sub-cultured from agar to Sabouraud dextrose broth and maintained on a rotating wheel. Under these conditions, the mycelium grows as a ball-like cluster and releases single-celled spores into the broth. Only the spores were used for growth inhibition assays. *A. hydrophila* was isolated from diseased Wyoming toads by S.K.T. [22,36]. A second isolate from the American type culture collection (isolate 43408) was also tested. Both isolates were grown in nutrient broth (Difco 0003) (Difco Laboratories, Detroit, MI USA) at 30°C.

2.2. Growth inhibition assays

The growth inhibition assays were adapted from an assay described by Mor and Nicolas [50]. For growth inhibition of fungal cells, 5×10^4 cells in a volume of 50 μl of broth were plated in replicates of five or more in a 96-well microtiter plate with or without addition of 50 μl serial dilutions of each peptide in broth (Sigma Chemical, St Louis, MO, USA). Positive control wells received 50 μl of broth without peptide, and negative control wells (on a separate plate) received 50 μl of broth containing 0.4% paraformaldehyde [50]. Growth at 24, 48, 72, and 96 h (23°C) was measured as increased optical density at 492 nm with an ELISA plate reader. For growth inhibition studies of *A. hydrophila*, 50 μl of bacteria, at a concentration of 1×10^8/ml, were plated in replicates of five or more in a 96-well microtiter plate with or without addition of 50 μl of serial dilutions of each antimicrobial peptide in water. Positive control wells received 50 μl water, negative control wells (on a separate plate) received 50 μl of 0.4% paraformaldehyde in water [50]. Growth was measured at 24 and 48 h as increased optical density at 492 nm with an ELISA plate reader.

2.3. Peptides

Magainin I, magainin II, ranalexin, and dermaseptin were purchased from Sigma Chemical (St Louis, MO, USA). Magainin II, PGLa, and CPF were a generous gift of Magainin Pharmaceuticals (Plymouth Meeting, PA, USA). The CPF used in these experiments is a variant with residue 20 changed from Met to Leu. All peptides were dissolved in glass distilled water, filter sterilized, and frozen in small aliquots at high concentration (usually 1.0 mg/ml) at −70°C and used at various dilutions for culture. For growth inhibition of *A. hydrophila*, the peptides were diluted in sterile distilled water. For the fungal growth inhibition assays, the peptides were diluted in broth.

3. Results

3.1. Peptide-induced growth inhibition of B. dendrobatidis

Growth of *B. dendrobatidis* was significantly inhibited at 48 h of culture at concentrations of about 50–100 μM of the magainins (Fig. 1(a) and (b)), 12.5 μM of CPF (Fig. 1(c)), and 100 μM of PGLa (Fig. 1(d)). Similarly, dermaseptin, at about 23 μM (Fig. 1(e)), and ranalexin, at about 9 μM (Fig. 1(f)), significantly inhibited growth. Both mature fungal cells and flagellated zoospores were included in the cultures. Thus, the minimal inhibitory concentrations (MIC) shown for CPF, PGLa, dermaseptin, and ranalexin are indicative of complete growth inhibition of all stages of the life cycle (Fig. 1(c)–(f)). The relationship of $O.D._{492}$ to relative cell numbers can be expressed as follows: When mature cells are plated at a concentration of 5×10^5/ml, the initial $O.D._{492}$ readings are about $1–2 \times 10^{-2}$. After culture for 48 h, the $O.D._{492}$ has increased to about $8–10 \times 10^{-2}$. This corresponds to a concentration of mature cells of about 5×10^6/ml. Growth inhibition that results in a decrease of $O.D._{492}$ to $4–5 \times 10^{-2}$ represents a loss of about half of the cells (data not shown).

3.2. Peptide-induced growth inhibition of B. ranarum

Growth of *B. ranarum* was significantly inhibited at 48–96 h of culture by relatively low concentrations of magainin I (7.8 μM) (Fig. 2(a)), magainin II (2.0 μM) (Fig. 2(b)), CPF (3.1 μM) (Fig. 2(c)), PGLa (3.1 μM) (Fig. 2(d)), dermaseptin (22.8 μM) (Fig. 2(e)), and ranalexin (9.4 μM) (Fig. 2(f)). Although growth was inhibited at these lower concentrations, much higher concentrations were required to completely eliminate

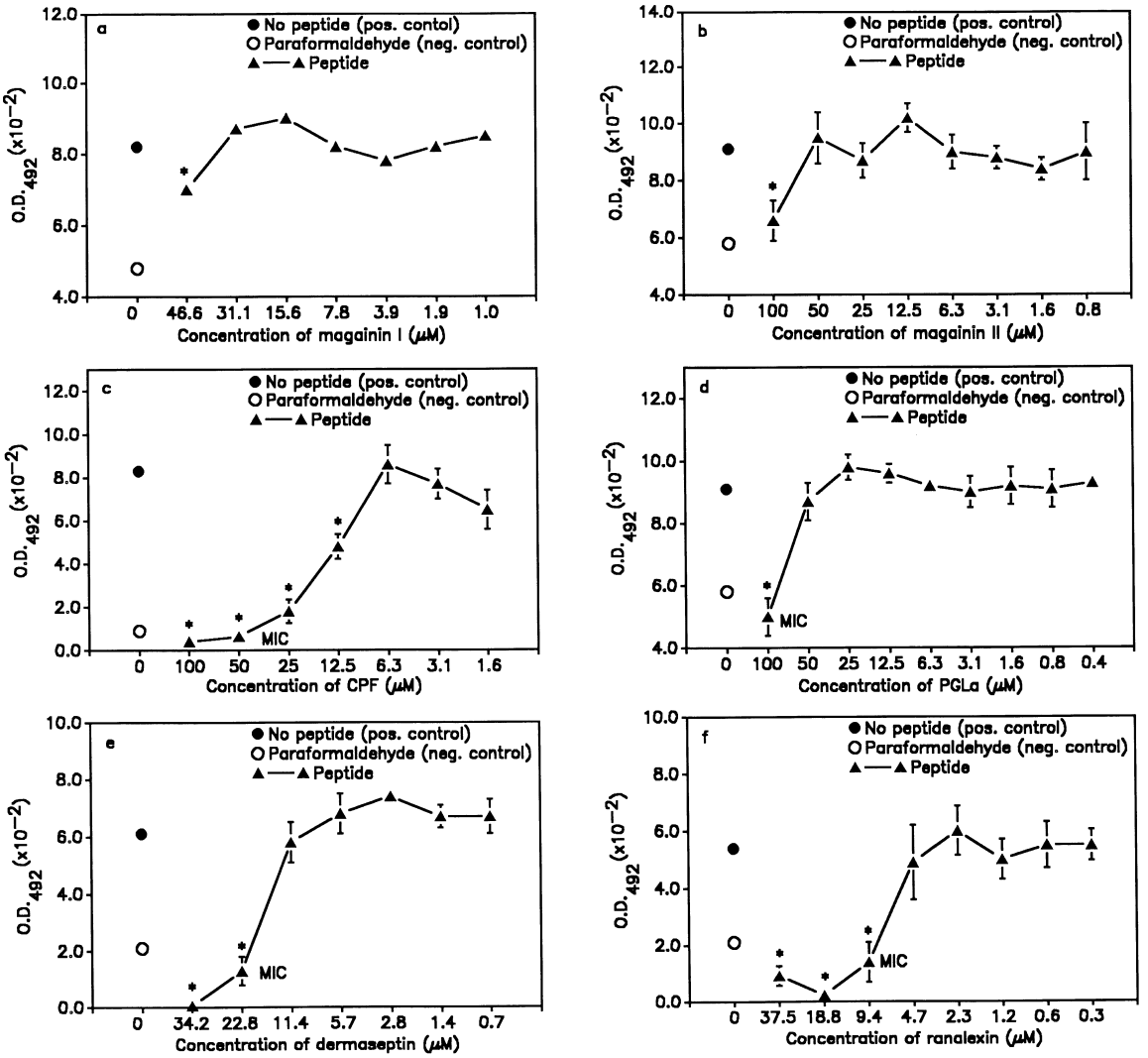

Fig. 1. Growth inhibition of *B. dendrobatidis* at 48 h by: (a) magainin I; (b) magainin II; (c) CPF; (d) PGLa; (e) dermaseptin; and (f) ranalexin. Each data point represents the mean ± SE of replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Significantly less growth than positive controls (one-tailed Student's *t*-test, $P \leq 0.025$). The results are representative of four assays of magainin I, 11 assays of magainin II, four assays of CPF, five assays of PGLa, three assays of dermaseptin, and three assays of ranalexin. Minimal inhibitory concentration (MIC) is the lowest concentration at which no growth was detected.

growth of both *Batrachochytrium* (Fig. 3(a)) and *Basidiobolus* (Fig. 3(b)) during 4 days of culture. For example, although the growth of *Batrachochytrium* and *Basidiobolus* was significantly inhibited at 24 and 48 h by 12.5 μM of CPF or magainin II, significant growth was detectable at later time points (Fig. 3(a) and (b)). This pattern was also observed with other peptides and suggests that some cells can escape the effects of lower concentrations of antimicrobial peptides.

3.3. Lack of peptide-induced growth inhibition of A. hydrophila

In contrast to their effects on both fungal pathogens, none of the peptides tested inhibited growth of an isolate

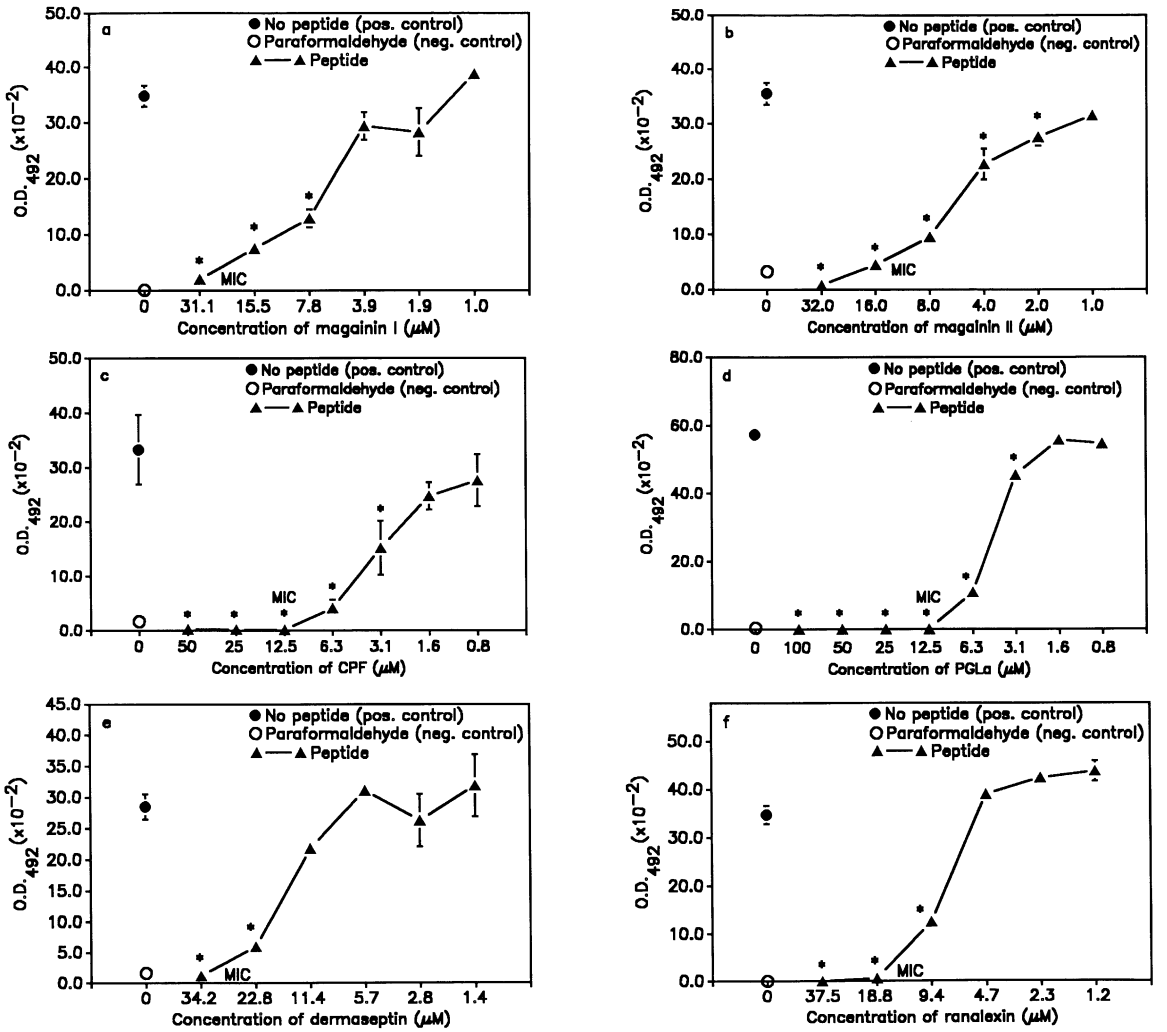

Fig. 2. Growth inhibition of *B. ranarum* by: (a) magainin I and (b) magainin II at 48 h; (c) CPF and (d) PGLa at 96 h; (e) dermaseptin and (f) ranalexin at 48 h. Each data point represents the mean ± SE of replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Significantly less than positive controls (one-tailed Student's *t*-test, $P \leq 0.05$). The results are representative of three assays of magainin I, six assays of magainin II, one assay of CPF, two assays of PGLa, two assays of dermaseptin, and two assays of ranalexin. MIC is the lowest concentration at which no growth was detected.

of *A. hydrophila* collected from diseased Wyoming toads (*B. baxteri*) [22,36]. Magainin II, PGLa, and CPF were tested at concentrations of 1.6–100 μM. Magainin I was tested at 0.2–75 μM, dermaseptin at 0.2–34.0 μM, and ranalexin at 0.3–18.8 μM (data not shown). A second isolate from the American type culture collection (isolate 43408) was also resistant to magainin I, magainin II, ranalexin, and dermaseptin at concentrations of about 0.2–18.8 μM (data not shown).

3.4. Synergistic effects of magainin II and PGLa

Because antimicrobial peptides are naturally produced and released in the skin as mixtures of peptides [38], we tested, in combination, the activity of two peptides derived from the skin of *X. laevis*. When tested individually against *B. dendrobatidis*, both magainin II and PGLa inhibited growth only at a relatively high concentration (100 μM). However, when added

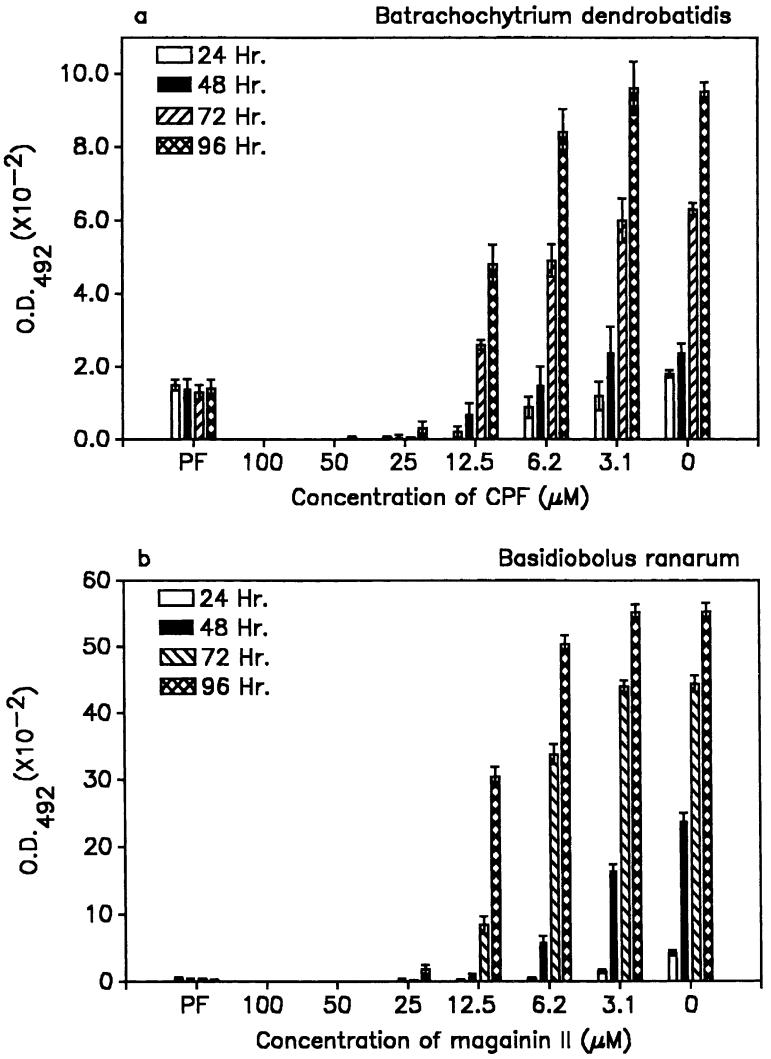

Fig. 3. Growth inhibition of (a) *B. dendrobatidis* by CPF and (b) *B. ranarum* by magainin II over a period of 96 h. PF = 0.4% paraformaldehyde. Each vertical bar represents the mean ± SE of replicate wells.

together to a culture of *B. dendrobatidis* (at a ratio of 1:1), inhibition was synergistic. That is, concentrations as low as 6.25 μM of each peptide (12.5 μM total) were significantly inhibitory (Fig. 4(a)). When tested individually against *B. ranarum*, magainin II inhibited at a concentration of about 12.5 μM, and PGLa inhibited at a concentration of about 3.1 μM. However, when added together at a 1:1 ratio in culture, they were completely inhibitory at a concentration of 0.4 μM of each peptide (0.8 μM total) (Fig. 4(b)). The peptides failed to inhibit growth of *A. hydrophila*, alone or in combination, at concentrations as high as 50 μM of each peptide (100 μM total) (Fig. 4(c)).

4. Discussion

All of the organisms examined in this study are able to infect amphibian skin. Thus, the skin as a defensive barrier is central to protection from these pathogens. We have shown here that representative antimicrobial peptides derived from the skin of three species of

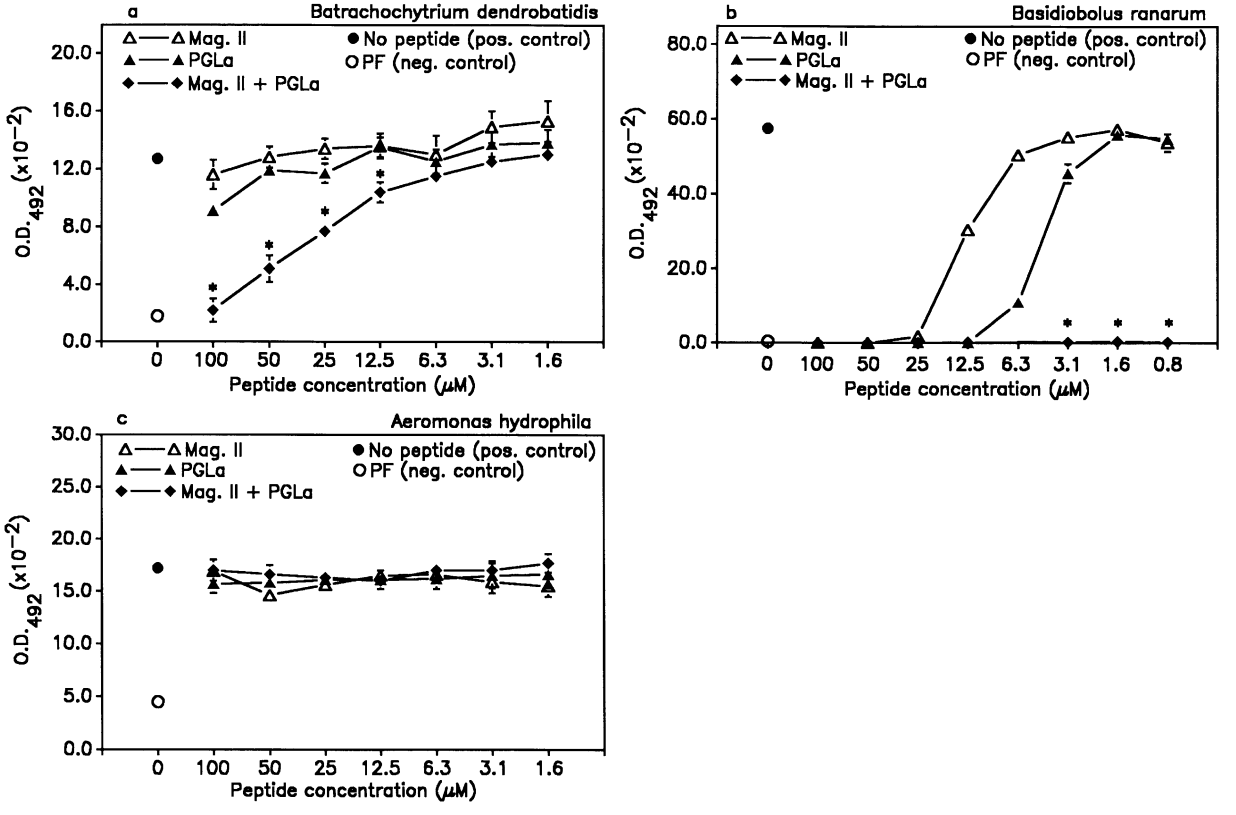

Fig. 4. Synergistic growth inhibition by magainin II and PGLa of (a) *B. dendrobatidis* and (b) *B. ranarum* at 96 h of culture, but not (c) *A. hydrophila* at 48 h of culture. Each data point represents the mean ± SE of replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Concentrations at which synergistic effects were observed. Synergism is defined as the concentration at which a 1:1 molar ratio of both peptides is more inhibitory than an equal concentration of either peptide alone (one-tailed Student's *t*-test, $P \leq 0.025$).

frogs can kill or inhibit the growth of both fungal species but not *Aeromonas.* Although each peptide varied in its pattern of effectiveness, at least one peptide from each species was effective against both fungi at a concentration of about 10–20 μM. Almost nothing is known about the concentrations of antimicrobial peptides present on the skin of healthy resting amphibians, but the concentration released in skin secretions following electrical stimulation or treatment with adrenergic agents can exceed 1 mg/ml ([51]; M. Zasloff, unpublished observation). The mechanism of action of a limited number of peptides has been studied in detail. In general, the cationic antimicrobial peptides, such as magainin, are thought to act by binding to the cell membrane and self-associating to form a membrane channel or pore (reviewed in Ref. [52]).

Magainin II and PGLa, derived from *X. laevis*, acted synergistically against the fungi, but not *Aeromonas*. The mechanism for this synergism between magainin II and PGLa has been studied using artificial phospholipid bilayers surrounding fluid droplets (liposomes). The synergism was due to the formation of a two-peptide complex in which the peptides were represented at a 1:1 ratio in the artificial membrane. This association resulted in rapid and stable membrane pore formation. Magainin alone formed pores slowly. PGLa alone formed unstable pores [53]. It is likely that a mixture of peptides would be more effective on the skin surface than individual peptides. Thus, at least some species of amphibians have antimicrobial peptide defenses in the skin that should serve as a first line of defense against fungal pathogens. Furthermore, a recent study demonstrating that peptide secretions were increased after exposure of frogs to environmental pathogens supports a role for antimicrobial skin peptides in defense against such microbes [54].

Although growth of *Batrachochytrium* and *Basidiobolus* was inhibited, growth of *A. hydrophila*, a frequent resident on the skin of healthy frogs [32], was not inhibited. Preliminary experiments also suggest that the magainins do not reduce infectivity of a salamander iridovirus (*Ambystoma tigrinum* Virus), when applied to high titer virus preparations at 50 μg/ml (about 15 μM) (T. O'Connor and E. Davidson, personal communication). In contrast, both esculentin-2P and ranatuerin-2P, derived from the skin of *R. pipiens*, effectively inhibit plaque formation by another iridovirus (frog virus 3) when used at a concentration of 50 μM (V.G. Chinchar and L. Rollins-Smith unpublished observation). More complete studies of the effectiveness of these antimicrobial peptides in defense against iridovirus infections are underway. Presumably, other components of amphibian immune defenses (such as antibodies and T-cell mediated responses) must be responsible for defense against certain bacterial and viral pathogens resistant to antimicrobial peptides.

None of the peptides tested in this study were derived from species that are currently in decline, and very little is know about the antimicrobial peptide defenses of species that are undergoing declines. However, caerin 1.1, a major defensive peptide found in the skin of Australian tree frogs (*Litoria splendida*, *Litoria gilleni*, and *Litoria caerulea*) can inhibit *B. dendrobatidis* zoospore motility and encystment of sporangia at concentrations of 50–100 μg/ml (L. Berger, J. Bowie, and A. Hyatt, personal communication). One of these species, *L. caerulea,* is experiencing mass mortality due to chytridiomycosis [17]. Future studies must determine whether declining species have less effective antimicrobial peptide defenses against *Batrachochytrium* than thriving species. If healthy members of declining species are shown to possess a repertoire of antimicrobial peptides that are effective against this pathogen, it will be critical to determine whether these defenses are impaired by environmental factors (such as UV-B or toxic chemicals). Environmental factors may, directly or indirectly, inhibit optimal synthesis or secretion of peptides. For example, stress that elevates glucocorticoids may inhibit peptide production and release [54–56]. Additional research is urgently needed to understand the nature of the immune defenses in amphibian skin, the mechanisms used by each pathogen to subvert those defenses, and the effects of environmental factors on the interplay of pathogen and host defenses.

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