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Hybridization between the African clawed frogs *Xenopus laevis* and *Xenopus muelleri* (Pipidae) increases the multiplicity of antimicrobial peptides in skin secretions of female offspring

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ABSTRACT

Peptidomic analysis was used to compare the distribution of host-defense peptides in norepinephrine-stimulated skin secretions from laboratory-generated female F1 hybrids of the common clawed frog *Xenopus laevis* (Daudin, 1802) and Mueller's clawed frog *Xenopus muelleri* (Peters, 1844) with the corresponding distribution in skin secretions from the parent species. A total of 18 peptides were identified in secretions from the hybrid frogs. Eleven peptides (magainin-1, magainin-2, CPF-1, CPF-3, CPF-4, CPF-5, CPF-6, CPF-7, XPF-1, XPF-2, and PGLa) were identified in secretions of both the hybrids and *X. laevis*. Four peptides (magainin-M1, XPF-M1, CPF-M1, and tigerinin-M1) were previously found in skin secretions of *X. muelleri* but magainin-M2 and CPF-M2 from *X. muelleri* were not detected. Three previously undescribed peptides (magainin-LM1, PGLa-LM1, and CPF-LM1) were purified from the secretions of the hybrid frogs that were not detected in secretions from either *X. laevis* or *X. muelleri*. Magainin-LM1 differs from magainin-2 from *X. laevis* by a single amino acid substitution ($Gly^{13} \rightarrow Ala$) but PGLa-LM1 and CPF-LM1 differ appreciably in structure from orthologs in the parent species. CPF-LM1 shows potent, broad-spectrum antimicrobial activity and is hemolytic. The data indicate that hybridization increases the multiplicity of skin host-defense peptides in skin secretions. As the female F1 hybrids are fertile, hybridization may represent an adaptive strategy among *Xenopus* species to increase protection against pathogenic microorganisms in the environment.

1. Introduction

The clawed frogs of the genus *Xenopus* within the family Pipidae currently comprise 19 well characterized species although several unnamed species have been reported (Kobel et al., 1996; Frost, 2011). All are found in Africa south of the Sahara. The genus has a complex evolutionary history involving both bifurcating and reticulating modes of speciation (Kobel, 1996; Evans et al., 2004; Evans, 2008). Allopolyploidization events, in which two species hybridize and the descendant inherits the complete genome of both ancestors, have given rise to tetraploid, octoploid, and dodecaploid species with no extant *Xenopus* species retaining the diploid status that is thought to be related to the ancestral state existing prior to one or more whole genome duplications (Kobel and Du Pasquier, 1991). At this time, the ten tetraploid *Xenopus* species have been divided into three species groups on the basis of similarities in morphology, advertisement calls, and/or nucleotide sequences of mitochondrial genes: the *laevis* group includes *X. laevis*, *X. gilli*, *X. largeni*, *X. petersii*, and *X. victorianus*; the *muelleri* group includes *X. muelleri*, *X. borealis*, and *X. clivii*; and the *fraseri* group includes *X. fraseri* and *X. pygmaeus* (Kobel et al., 1996; Evans et al., 2004). It has been proposed that the seven extant octoploid species arose from three distinct allopolyploidization events (Evans, 2008; Evans et al., 2011). Thus, *X. lenduensis* and *X. vestitus* share a common tetraploid ancestor; *X. amieti*, *X. andrei*, and *X. boumbaensis* form a second group; and *X. itombwensis* and *X. wittei* constitute a third group. Further allopolyploidizations within the second group have given rise to the dodecaploid species *X. longipes* and *X. ruwenzoriensis*.

The skins of frogs from the genus *Xenopus* have proved to be a rich source of host-defense peptides with antimicrobial and anti-inflammatory activities [reviewed in (Conlon et al., 2012)]. The magainins were isolated from *X. laevis* skin more than 25 years ago, (Giovannini et al., 1987; Zasloff, 1987) and analysis of skin

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secretions from this species led to the isolation and characterization of several other peptides that show therapeutic potential as anti-infective agents (Zhang and Falla, 2010). These include peptide glycine-leucine-amide (PGLa) and multiple structurally-related peptides that are derived from the post-translational processing of the biosynthetic precursors of caerulein [termed caerulein-precursor fragment (CPF)] and xenopsin [termed xenopsin-precursor fragment (XPF)] (Gibson et al., 1986; Soravia et al., 1988). More recently, antimicrobial peptides belonging to the magainin, PGLa, CPF, and XPF families have been isolated from norepinephrine-stimulated skin secretions from *X. amieti* (Conlon et al., 2010), *X. andrei* (Mechkarska et al., 2011b) , *X. borealis* (Mechkarska et al., 2010), *X. clivii* (Conlon et al., 2011), *X. lenduensis* (King et al., 2012), *X. muelleri* and an incompletely characterized species from West Africa provisionally designated *X. muelleri* West (Mechkarska et al., 2011a), *X. petersii* (King et al., 2012), and *X. pygmaeus* (King et al., 2012).

There have been relatively few reports of natural hybridization between *Xenopus* species in the wild. Extensive interbreeding has been described only for *X. laevis* and the seriously endangered *X. gilli* (Picker, 1985). Hybrids between *X. borealis* and *X. victorianus* in western Kenya have also been described (Yager, 1996). Areas of sympatry between *X. laevis* and *X. muelleri* are few which mitigates against interbreeding but the two species were found in the same bodies of water in Mpumalanga province of South Africa and natural hybrids were characterized both biochemically and morphologically and from their distinctive advertisement calls (Fischer et al., 2000). In contrast, many *Xenopus* species can be hybridized in the laboratory and gametic incompatibilities are generally found to be absent (Kobel et al., 1996). In such interspecies crosses, the males are consistently sterile and the females are fully or partially fertile. As *Xenopus* females are heterogametic and males are homogametic, it has been pointed out that this genus represents an exception to Haldane's rule that the heterogametic sex typically suffers the greater dysfunctional effects of hybridization (Malone et al., 2007).

The generation of *X. laevis*×*X. muelleri* hybrids in the laboratory has been described and microarray analysis has been used to study gene expression in the testes of hybrid males (Malone et al., 2007) and in the ovary of hybrid females (Malone and Michalak, 2008). These animals have been made available to the investigators and the aim of the present study was to use peptidomic analysis (reversed-phase HLPC coupled with electrospray mass spectrometry) to compare the host-defense peptides in norepinephrine-stimulated skin secretions from female *X. laevis*×*X. muelleri* F1 hybrids with the corresponding peptides from the parent species. The peptides described in this study are classified according to the terminology used previously for peptides from the *X. laevis* (Gibson et al., 1986; Zasloff, 1987). The magainin, PGLa, CPF, and XPF peptide families are recognized. The term tigerinin refers to the family of cyclic peptides first identified in the Asian frog *Hoplobatrachus tigerinus* (Sai et al., 2001). Orthologs from *X. muelleri* are designated M and the novel peptides identified in the hybrids are denoted by LM. Paralogs are differentiated by numerals e.g. CPF-1 and CPF-2.

2. Materials and methods

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. *X. laevis*×*X. muelleri* F1 hybrids were produced as previously described (Malone et al., 2007). The female parents were *X. laevis* originating from the Cape region of South Africa and the male parents were *X. muelleri* originating from the Nkambeni area of Swaziland. Female hybrids ($n=3$; 5–6 years old; weights 43–48 g) were injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body mass) and placed in a solution (100 mL) of collecting buffer (50 mM sodium chloride-25 mM sodium acetate, pH 7.0) for 15 min. The frogs were removed and the collection solution was acidified by addition of concentrated hydrochloric acid (1 mL) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions from each group were pooled and separately passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (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).

A single adult female specimen of *X. laevis* (weight 263 g) was supplied by Xenopus Express Inc. (Brooksville, FL, USA) and housed in the vivarium at U.A.E. University. Skin secretions were obtained and partially purified on Sep-Pak cartridges using the same protocol used for the hybrids. The collection of skin secretions from *X. muelleri* has been described previously (Mechkarska et al., 2011a).

2.2. Peptide purification

The pooled skin secretions from the hybrid frogs, 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, USA) 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 (100 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 (1.0 cm×25 cm) Vydac 214TP510 (C-4) column and a (1.0 cm×25 cm) Vydac 208TP510 (C-8) 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.

Skin secretions from *X. laevis* (20% of the total amount collected) were subjected to the same chromatographic procedures used for purification of the peptides from the hybrid frogs. The purification of antimicrobial peptides from *X. muelleri* skin secretions has been described previously (Mechkarska et al., 2011a).

2.3. Structural characterization

Electrospray-ionization mass spectrometry was carried out using an Agilent 6310 Series ion trap mass spectrometer as previously described (Zahid et al., 2011). The polarity of the mass spectrometer was set to positive and the scan range was from 200 to 2200 m/z with maximum accumulation time of 300 ms. The capillary voltage was set to −3500 V, the skimmer voltage was 36.3 V and the trap drive was 74.2 V. The flow of drying gas was set to 10 l/min, the nebulizer gas pressure was set to 70 psi, and the drying temperature was 350 °C. The accuracy of mass determinations was ±0.05%. The primary structures of the novel peptides were determined by automated Edman degradation using a model 492 Procise sequenator (Applied Biosystems, Foster City, CA, USA).

2.4. Peptide synthesis

PGLa-LM1 and CPF-LM1 were supplied in crude form by GL Biochem. Ltd (Shanghai, China) and were purified to near homogeneity (>98% purity) by reversed-phase HPLC on a (2.2 cm×25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 60 min and the