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SCIENCE DIRECT
General and Comparative Endocrinology 146 (2006) 242–250
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Developmental and triiodothyronine-induced expression of genes encoding preprotemporins in the skin of Tago's brown frog *Rana tagoi*

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Received 5 October 2005; revised 15 November 2005; accepted 25 November 2005
Available online 5 January 2006

Abstract

Using RT-PCR, two cDNAs encoding preprotemporins were cloned from a total RNA preparation of the skin of Tago's brown frog *Rana tagoi*. Preprotemporin-1TGa cDNA directs the synthesis of temporin-1TGa (FLPILGKLLSGIL.NH_2) previously isolated from *R. tagoi* skin. Preprotemporin-1TGb cDNA directs the synthesis of a novel 16-amino-acid-residue peptide (AVDLAKIANKVLSSLF.NH_2) that, atypically for members of the temporin family, inhibits the growth of Gram-negative bacteria more effectively than Gram-positive bacteria. Preprotemporin-1TGa mRNA and preprotemporin-1TGb mRNA were not detected in skin prior to the onset of metamorphosis (stage 35) but the levels of the transcripts increased markedly during metamorphosis reaching a maximum at stage 38. Exposure of adult animals to 10^{-8} M triiodothyronine (T_3) for 72 h enhanced expression of the preprotemporin-1TGb gene (approximately threefold) but did not significantly change the level of expression of the preprotemporin-1TGa gene. Exposure of the animals to 10^{-8} M T_3 and 10^{-6} M bisphenol A, an endocrine disrupting chemical that potently inhibits the action of thyroid hormones (THs), reduced expression of the preprotemporin-1TGb gene by 10-fold and the preprotemporin-1TGa gene by threefold. We propose that T_3-stimulated synthesis of antimicrobial peptides is important in protecting the animal against microorganisms, particularly at metamorphosis and during skin moulting, but environmental pollutants can inhibit peptide synthesis and render the animal susceptible to invasion by pathogens.

Keywords: Frog skin; Temporin; Antimicrobial peptide; Triidothryonine; Bisphenol A; Endocrine disruptor

1. Introduction

Peptides with antimicrobial activity play an important role in the innate immunity that predates adaptive immunity and constitutes the first-line defense against invading pathogens for a wide range of vertebrate and invertebrate species (Hoffmann et al., 1999). For at least part of their life cycle, anurans (frogs and toads) are confined, of necessity, to a warm and moist environment that is conducive to the growth of potentially harmful bacteria and fungi. It is not surprising, therefore, that these animals have developed over the course of evolution a system of host defense that protects the skin from invasion by pathogenic microorganisms. For many, but by no means all, anuran species, the synthesis of polypeptides with a broad spectrum of antimicrobial activity in the granular glands that first appear in the skin at metamorphosis is an important feature of this defense strategy. The peptides are released into skin secretions, often in very high concentrations, in a holocrine manner upon stress or injury as a result of contraction of myocytes surrounding the glands (Conlon et al., 2004; Simmaco et al., 1998). Skin secretions generally contain multiple antimicrobial peptides with distinct spectra of activity, and it has been speculated that this molecular diversity is important in protecting the animal from invasion by a wide array of different microorganisms (Mor et al., 1994).

The factors that regulate the synthesis of antimicrobial peptides in the skins of frogs are incompletely understood.

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0016-6480/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2005.11.015

Induction of peptide synthesis in response to a bacterial challenge has been demonstrated in the case of *Rana sylvatica* (Matutte et al., 2000) and *Rana esculenta* (Mangoni et al., 2001). Glucocorticoid treatment of *R. esculenta* inhibits the transcription of all genes encoding antibacterial peptides by inducing the synthesis of IκBα, an inhibitor of NF-κB-regulated transcription (Simmaco et al., 1997). There is evidence for an involvement of THs in the regulation of antimicrobial peptide gene expression. In *Xenopus laevis* tadpoles, patterns of expression of the genes encoding the precursors of the magainins and an antimicrobial peptide with amino terminal glycine and carboxy terminal leucinamide (PGLa) were studied at various developmental stages (Reilly et al., 1994). These precursor gene transcripts increase dramatically at the onset of metamorphosis (between stages 58 and 60), and the mature peptides are first detected in the skin at stage 60. These facts suggest a correlation between the antimicrobial peptide gene expression and the THs. THs play essential roles in the initiation, progression, and completion of amphibian metamorphosis and their concentrations in plasma reach a peak at the pro-metamorphic stages in parallel with the increase in antimicrobial peptide gene expression (Shi, 2000).

The aim of the present study is to investigate the effects of exogenous triiodothyronine (T_3) and of the increased circulating concentrations of TH during metamorphosis on the expression of genes encoding antimicrobial peptides in the skin of Tago's brown frog *Rana tagoi*. In particular, the expression of the gene encoding preprotemporin-1TGa, the biosynthetic precursor of the previously identified antimicrobial peptide, temporin-1TGa (Conlon et al., 2003), and the gene encoding preprotemporin-1TGa, the precursor of a novel 16-amino-acid-residue antimicrobial peptide, temporin-1TGb, was studied. It has previously been shown that the environmental pollutant, bisphenol A (BPA; 4,4′-isopropylidenediphenol) inhibits both spontaneous and TH-induced metamorphosis of *Xenopus* tadpoles by suppression of thyroid hormone receptor (TR) β gene expression (Iwamuro et al., 2003). Consequently, the effect of this agent on T_3-induced expression of the preprotemporin genes in adult *R. tagoi* skin was also investigated.

2. Materials and methods

2.1. Animals

Adult *R. tagoi* specimens (3–5 cm body length) were captured in Ichihara City, Chiba Prefecture, Japan, in July 2004. The animals were kept at 23 °C under 12 h light–12 h dark illumination conditions in plastic tanks equipped with a few small stones and containing an appropriate volume of dechlorinated water. A mass of oocytes were obtained in the same place at which the adult frogs were captured. The oocytes, embryos, and tadpoles were also kept in dechlorinated water under the same conditions. The embryos and tadpoles were staged according to the system described for *Rana brevipoda* (Iwasawa and Morita, 1980). All experiments were approved by Toho University Bioethics and Animal Ethics Committee and were carried out by authorized investigators.

2.2. T_3 and BPA treatment

3,3′,5-Triiodo-L-thyronine (T_3) was dissolved in 10 mM NaOH to produce a 10^{-4} M stock solution and diluted in dechlorinated water to 10^{-8} M. Adult *R. tagoi* specimens ($n = 3$) were immersed in a solution (10^{-8} M T_3; 300 mL) of T_3 for 72 h in a plastic container (140 × 180 × 60 mm) equipped with a fiber base. Three control specimens were kept under identical conditions except that the solution used for immersion did not contain T_3. The animals were anesthetized by immersion in ice and were sacrificed by decapitation. Skin was removed immediately and extracted as described. It has previously been shown in *Rana catesbeiana* that immersion of frogs in a solution of TH raises the concentration in the circulation (Wright and Alves, 2001).

In a second series of experiments, four groups of adult specimens (three animals in each group) were immersed in a solution of (A) 10^{-6} M BPA, (B) 10^{-5} M BPA, (C) 10^{-6} M BPA + 10^{-8} M T_3, and (D) 10^{-5} M BPA + 10^{-8} M T_3 for 72 h under the same conditions.

2.3. cDNA cloning by RT-PCR

Total RNA was extracted from the skin of each frog by the acid–guanidine isothiocyanate–phenol–chloroform procedure (Chomczynski and Sacchi, 1987). The open reading frame (ORF) of the temporin-1TGa precursor protein and part of elongation factor-1α (EF1α) cDNA were amplified by using a One-Step RT-PCR Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. Briefly, 100 ng of total RNA and a set of primers were incubated at 50 °C for 30 min for the reverse transcription and then at 95 °C for 15 min for the denaturation of the reverse transcriptase. Subsequently, PCR was performed with a thermal cycler under the following conditions: 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C for 35 cycles (for preprotemporin-1TGa) or 30 cycles (for EF1α). Finally, the reaction mixtures were kept at 72 °C for 7 min to complete extension of the DNA. The sense primer (5′-AACTGAACCACCCGAGCCCAAA-3′) and antisense primer (5′-AGATGATTTCCAATTCCAT-3′) for the preprotemporin-1TGa were designed from conserved regions of the 5′- and 3′-untranslated regions (UTRs) of *Rana temporaria* preprotemporins B, G, and H (Simmaco et al., 1996) (GenBank Accession Nos. Y09393, Y06395, and Y09394, respectively). The sense primer (5′-ACATGAGGCAGACTGTTGCC-3′) and antisense primer (5′-TTCCTTCTGAAGCTCTTGCG-3′) for the amplification of *R. tagoi* EF1α cDNA were designed according to the nucleotide sequence of *Xenopus* EF1α (GenBank Accession No. M25504). The RT-PCR products were separated by electrophoresis on a 1.5% agarose gel. The amplified DNAs with the appropriate sizes were excised and purified by a Wizard SV gel and PCR cleanup system (Promega, Madison, WI, USA) and subcloned into a pSTBlue-1 vector with an Acceptor Vector Kit (Novagen, Darmstadt, Germany). Nucleotide sequence analysis was performed on both strands by Biomatrix Company (Chiba, Japan).

2.4. Semiquantitative analysis of PCR products

Analysis was carried out by a modification of a procedure previously described (Iwamuro et al., 2003). RT-PCR products (10 μL aliquots) were separated on 1.5% agarose gel and stained with ethidium bromide. Fluorescent images of the amplified DNAs were recorded with a Kodak digital image analyzer (Eastman Kodak, Rochester, NY, USA) and concentrations were calculated semiquantitatively with a computer software NIH image system using Takara DNA ladder markers (Ohtsu, Japan) (100 ng/band) as a reference standard. The optimum conditions for RT-PCR were determined by changing the initial amounts of total RNA and/or the number of PCR cycles. When the number of PCR cycles was fixed at 28 and the amount of template RNA was in the range 6.25–400 ng, the relative intensity of the band corresponding to preprotemporin-1TGa was within the linear range (Fig. 1A). For 28 cycles and the amount of template RNA in the range 25–400 ng, the intensity of the preprotemporin-1TGb band was within the linear range (Fig. 1B). Similarly, when the number of PCR cycles was fixed at 36 and the amount of template RNA was in the range 25–400 ng, the relative intensity of the band corresponding to EF1α was