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Multiple bombesin-like peptides with opposite functions from skin of *Odorrana grahami*

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Abstract

Bombesin-like peptides (BLPs) are a family of neuroendocrinic peptides that mediate a variety of biological activities. Three mature BLPs from the skin secretions of the frog *Odorrana grahami* were purified. Several bombesin-like peptide cDNA sequences encoding precursors of BLPs were identified from the skin cDNA library of *O. grahami.* This is the maximal diversity of BLPs ever found in animals. Five mature BLPs (B1–B5) based on the amino acid sequences derived from the cDNA cloning were synthesized. In the in vitro myotropic contraction experiment, all synthesized BLPs displayed a stimulating effect toward rat stomach strips, except B4 and B5 which showed the opposite effect, suggesting that certain BLPs may act as antagonists of bombesin receptors while most other BLPs act as agonists. This finding will facilitate the finding of novel bombesin receptors and novel ligands of bombesin receptors. The diversity of amphibian BLPs and their precursors were also analyzed and results suggest that amphibian BLPs and corresponding precursors of various sizes and processing patterns can be used as markers of taxonomic and molecular phylogenetics. The remarkable similarity of preproregions gives rise to very different BLPs and 3′-terminal regions in distantly related frog species, suggesting that the corresponding genes form a multigene family originating from a common ancestor. The diversification of BLP loci could thus be part of an evolutionary strategy developed by amphibian species as a result of shifts to novel ecological niches when environmental factors change rapidly.

Keywords: Bombesin; Amphibian; Diversity; *Odorrana grahami*; Molecular marker

Bombesin modulates a variety of biological activities in the gastrointestinal (GI) tract and central nervous system (CNS) in mammals, including smooth muscle contraction, secretion of GI hormones [1,2], and regulation of central homeostatic mechanisms [3,4]. Bombesin was first isolated from European frog *Bombina bombina* skin in 1971 [5]. Several forms of bombesin were subsequently identified and cloned from different *Bombina* species, such as [Leu^{13}]bombesin, [Phe^{13}]bombesin, and [Ser^{3},Arg^{10}Phe^{13}]bombesin from *B. variegata* and *B. orientalis* [6–8]. Bombesin-related peptides ranatensin and [Leu^{8}]phyllolitorin were isolated from the skin of *Rana* and *Phyllomedusa* species [9,10]. These forms of bombesin characterized only in amphibians are designated bombesin-like peptides (BLPs) [6]. Using a gastrin release bioassay, a 27-amino-acid peptide homologous to the carboxyl terminus of bombesin was isolated from porcine stomach and named gastrin-releasing peptide (GRP) [11]. Hence, GRPs were purified and characterized from many vertebrate taxa including birds [12], dogfish [13], trout [14], frogs [15], alligators [16], and goldfish [17]. Neuromedin B (NMB) is the second mammalian BLP that was originally

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isolated from porcine spinal cord [18]. Both GRP and NMB are widely distributed in GI tract and CNS [19,20]. All these BLPs share a similar C-terminal heptapeptide and can be divided into three distinct branches: the BLP branch composed of the peptides originally found in amphibian skin (bombesin, ranatensin, and phyllolitorin), the NMB branch, and the GRP branch [21].

Although multiple forms of bombesin have been found in amphibians, the diversity of BLPs and their precursors are still not explicit. In this report, the isolation, characterization, and molecular cloning of five novel BLPs from skin secretions of *Odorrana grahami* are presented. The different processing patterns from precursors of bombesin to mature bombesins were observed among different families of amphibians. The functional significance of bombesin-like peptides, phylogenetic variation was also checked by testing one of their characterized physiological activities, myotropic effects on isolated rat stomach strip.

Results and discussion

Purification of bombesin-like peptides

The supernatant of *O. grahami* skin secretions was fractionated into several peaks by Sephadex G-50 (result illustrated in Fig. 1A). Among all peaks obtained, only peak VI was detected in the contractile activity on isolated rat stomach strips. Hence, the peak VI was collected and applied to an RP-HPLC. More than 50 peaks were obtained (Fig. 1B). The peaks with contractile activity (marked by B1, B3, and B4) were collected.

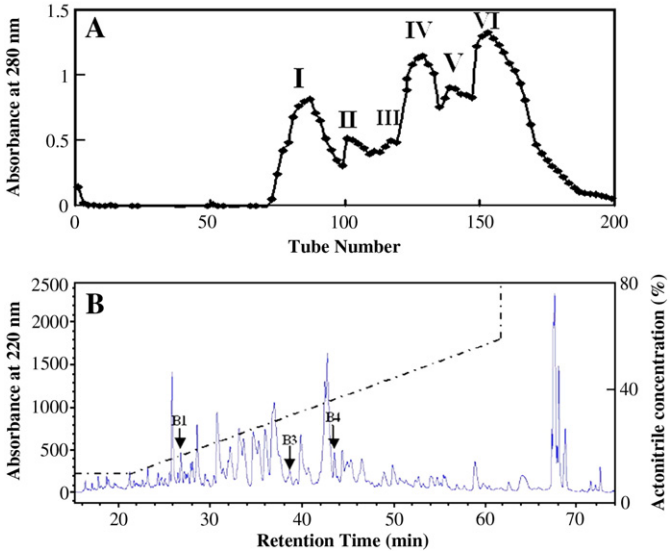

Fig. 1. Fractionation of *O. grahami* skin secretion. (A) Sephadex G-50 gel filtration of *O. grahami* skin secretion. *O. grahami* skin secretion was applied on a Sephadex G-50 (Superfine; Amersham Biosciences; 2.6 × 100 cm) column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The peak VI contractile activity on isolated stomach strips from Sephadex G-50 was further purified on a Hypersil BDS C_{18} RP-HPLC column (30 × 0.46 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. (B) The elution was performed with the indicated gradient of acetonitrile at a flow rate of 0.7 ml/min, and fractions were tested for contractile activity on isolated stomach strips. The purified peptides are indicated by B1, B3, and B4.

Structural characterization

The N termini of the purified peptides were demonstrated to be blocked in the amino acid sequence analysis by automated Edman degradation. After removing N-terminal pyroglutamic acid by treating with proglutamate amino peptidase, the amino acid sequences of the purified peptides were determined. Treating with carboxypeptidase Y did not lead to the release of free amino acids under conditions that free amino acids were released from a peptide with free C-terminal -COOH group. The result indicated that the C-terminal end of the peptide was amidated, which was further confirmed by fast atom bombardment (FAB) mass spectrometry analysis. The amino acid sequences of these three BLPs (indicated as B1, B3, and B4 in Fig. 1B) are pEREYRAPHWAIGHFM-NH_2 (B1), pERECRAPHWAIGHFM-NH_2 (B3), and pENTYRAPQWAVGHLM-NH_2 (B4), where pE is pyroglutamic acid. They are all composed of 15 amino acid residues, quite different from other amphibian BLPs.

All BLPs from *O. grahami* share the similar C-terminal sequence WAI/VGHF/LM-NH_2 but have variable N termini. All known BLPs from amphibians do not contain acidic amino acids in their N-terminal parts except for the BLP from *B. varigata*, $_{P}$EDSFGNQWARGHFM-NH_2 [22]. However, two BLPs that we isolated (B1 and B3) contained an acidic amino acid, glutamic acid, in their N-terminal parts. Interestingly, both B1 and B3 also possess a basic amino acid, arginine, at their second site of sequences that compensates the net charge caused by glutamic acid, while at the same site B4 contains neutral amino acids. Therefore, all three of these BLPs have the same total net charges although their amino acid components are different. Similarly, arginine compensates the net charge caused by Asp in $_{P}$EDSFGNQWARGHFM-NH_2, from *B. varigata*. It seems that the substitutions in these sites are coordinated. The significance of such substitutions will be described below.

cDNA cloning

Upon screening of a skin cDNA library, 42 clones containing inserts of around 350 to 360 bp were identified and isolated. Both strands of these clones were sequenced (Fig. 2). Twelve cDNA sequences (GenBank Accession Nos. DQ836109–DQ836120) encoding BLP precursors were obtained, and their deduced amino acid sequences are shown in Fig. 2. This is the maximal diversity of BLPs and their precursors ever found in animals. Three of the five mature BLPs have been purified from the skin secretions of *O. grahami* as described under Structural characterization.

The precursors of these BLPs are composed of 72 amino acids including an N-terminal signal peptide followed by an acidic peptide, a mature BLP, and a five-amino-acid C-terminal extension. They share highly conserved signal peptide, acidic peptide (SVCVEFMEDAGKLDKIDAF), and C-terminal

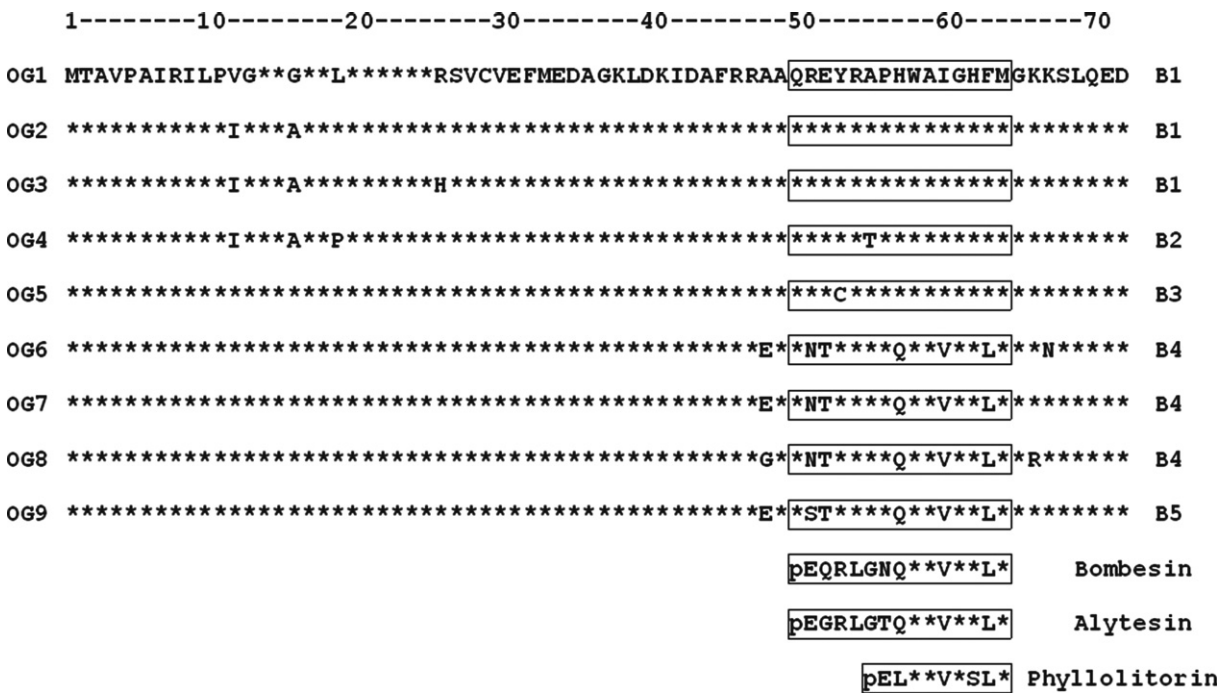

Fig. 2. Amino acid sequences deduced from the cDNA clones encoding bombesin-like peptides and their alignment with some BLPs. The clones are numbered as OGn at the left of the amino acid sequences of the precursors. The names of bombesin-like peptides are indicated at the right of the amino acid sequences of the precursors. Stars (*) indicate identical amino acid residues. The sequences of mature peptides are boxed. Bombesin and alytesin are from [5]; phyllolitorin is from [10].

extension parts (SLQED), while the regions of mature BLPs are quite different.

The precursors of BLPs isolated in our lab contain processing sites at their N and C termini similar to those of BLPs from other amphibian species, in which a dibasic amino acid cleavage sets off the N and C termini. An N-terminal glutamine provides the N-terminal pyroglutamyl residue and a C-terminal glycine provides the amide for the C-terminal methioninamide. However, no dibasic site but a monobasic site was observed in the C terminus of precursor B4 (Fig. 2). In addition, two processing sites occurred in all precursors of the BLPs. These BLPs are cleaved from their precursors at its N terminus following Ala–

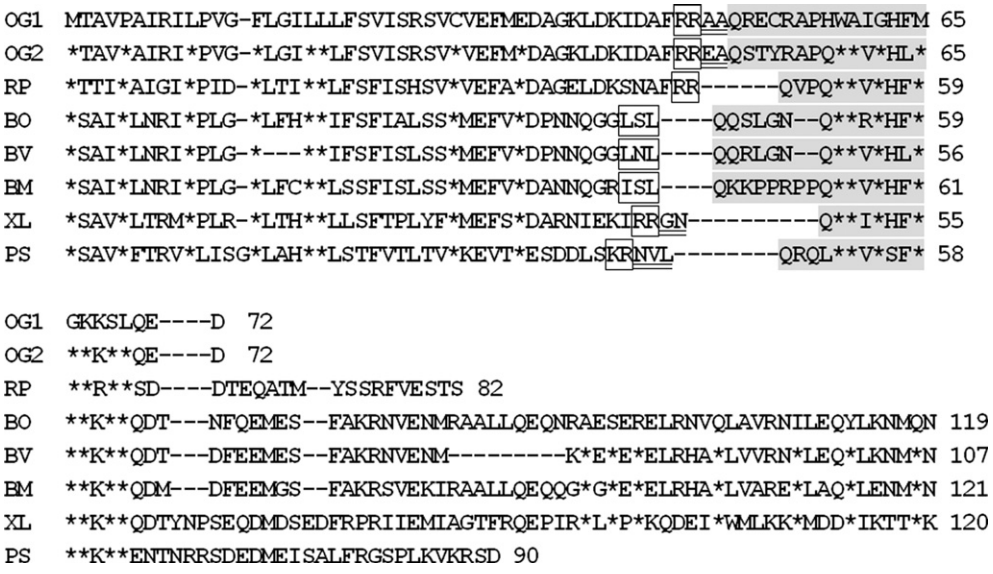

Fig. 3. Sequence comparison of the precursors of bombesin-like peptides from different amphibian families. OG, *Odorrana grahami* (Ranidae) [this study]; RP, *Rana pipiens* (Ranidae) [19]; PS, *Phyllomedusa sauvagei* (Rhacophoridae) [21]; BO, *Bombina oriential* (Discoglossidae) [15]; BV, *Bombina varigata* (Discoglossidae) [26]; BM, *Bombina maxima* (Discoglossidae) [23]; XL, *Xenopus laevis* (Pipidae) [24]. Stars (*) indicate the identical amino acid residues. The sequences of mature bombesin-like peptides are shaded. The processing sites of the precursor for releasing of mature BLPs are boxed. The second processing site is double underlined in some sequences. Gaps (-) have been introduced to optimize the sequence homology.

Ala, Glu–Ala, or Gly–Ala residues, followed by the dibasic processing site (Fig. 3).

The overall structures of precursors reported here are distinctively different from those of other precursors of amphibian BLPs (Fig. 3). Four different families of BLP precursors from amphibians were collected, including *O. grahami* (Ranidae) [this study], *Rana pipiens* (Ranidae) [19], *Phyllomedusa sauvagei* (Rhacophoridae) [21], *B. oriential* (Discoglossidae) [15], *B. varigata* (Discoglossidae) [22], *B. maxima* (Discoglossidae) [23], and *Xenopus laevis* (Pipidae) [24]. Among the families, the sizes and processing patterns of these BLPs and their precursors differ significantly (72–82 for Ranidae, 90 for Rhacophoridae, 96–110 for Discoglossidae, and109 for Pipidae) but, within each family, the precursors share similar sizes and nearly the same sizes within genera. The processing patterns from precursor to mature BLP were also different between families. Although all BLPs have the same dibasic processing sites in their C-terminal parts, the processing sites in the N-terminal parts are fairly different (see Fig. 3). For precursors from Ranidae they are Arg–Arg and Ala–Ala or Glu–Ala, and for Rhacophoridae they are Lys–Arg and Gln–Val–Leu, suggesting that these BLPs are processed by trypsin-like and elastase-like proteinases. The processing site for Discoglossidae is Leu–Ser–Leu, Leu–Gln–Leu, or Ile–Ser–Leu, indicating that it is processed only by elastase-like proteinase. Arg–Arg and Gly–Gln are the processing sites for Pipidae, showing that the Pipidae is processed by the same proteinases as Ranidae and Rhacophoridae. Furthermore, it is easy to deduce from Fig. 3 that the diversity of BLP precursors of different amphibian families may result from C-terminal deletion of ancestor precursors. From all of the facts above, it appears that the precursor sizes and the processing patterns of amphibian BLPs may be used as markers of taxonomic and molecular phylogenetics.

Myotropic effects on the isolated rat stomach strip

To investigate the functional significance of structurally different BLPs, the myotropic effects of BLPs on the isolated rat stomach strip were assessed. The results showed that B1, B2, and B3 induced the contraction of isolated stomach strip as Phe^{13}-bombesin, while B4 and B5 inhibited such contraction (Fig. 4). As indicated in Fig. 4, B4 and B5 relaxed the muscle. This result is discrepant from that in a previous publication [23], which reports that all BLPs can induce the stomach muscle contraction. In this study, some BLPs that inhibit the contraction of stomach muscle were isolated and identified. They appear to be a kind of antagonist of corresponding bombesin receptors. Interestingly, the N-terminal tripeptides B1, B2, and B3 have three charged amino acid residues (pE^1-R^2-E^3 in B1, B2, and B3) while the N-terminal tripeptides B4 and B5 have just one charged amino acid residue (pE^1-N^2-T^3 in B4 and pE^1-S^2-T^3 in B5) as shown Fig. 2. The structural difference may contribute to the functional difference. C-terminal octapeptides B4 and B5 are identical to bombesin and alytesin [5] (Fig. 2). The C-terminal octapeptide has still low contractile activity on rat uterus [25] while mature B4 and B5 inhibit the contraction of

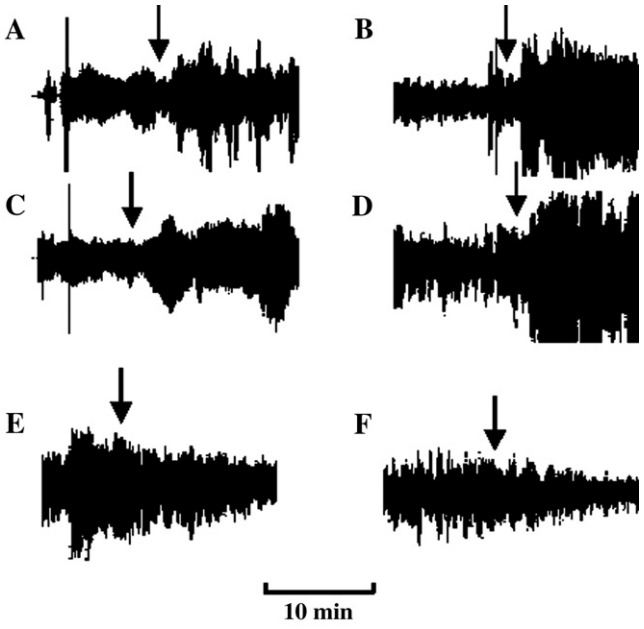

Fig. 4. Effect of bombesin-like peptides on isolated stomach strips. (A) Phe^{13}-bombesin; (B–F) B1–B5. The concentration of bombesin-like peptides is 5 μM in these experiments. The arrow (↓) indicates the time point when the tested sample is added. Phe^{13}-bombesin, B1, B2, and B3 induced the contraction of isolated stomach strips as other BLPs while B4 and B5 inhibited the contraction.

stomach strips. The functional difference of mature B4 and B5 and their C-terminal octapeptides suggests that the N termini of BLPs may exert effects on their myotropic responses on smooth muscles.

Several bombesin receptor families are identified: neuromedin B receptor (BB1), GRP receptor (BB2), the orphan receptor subtype (BB3), the bombesin receptor subtype (BB4), and the bombesin-like peptide receptor subtype 3.5 [23,24,26]. All display different affinities to different BLPs. Up to now, no endogenous ligand has been clearly identified for the subtype BB3 [27]. These data indicate that different BLPs have different physiological roles and distinct receptors. Some BLPs reported here contain uncommon N terminals, and they may act as a kind of antagonist of bombesin receptors or act on novel bombesin receptors. Further detailed analysis of the acting potency of these BLPs on different receptor subtypes would be highly interesting.

Conclusion

Five different BLPs from the skin of *O. grahami* were identified and synthesized. Their contractile effects on isolated rat stomach strip were assessed to investigate the functional significance of the structural diversification. BLPs isolated from *O. grahami* have divergent effects on isolated stomach strips. Three strengthen the contraction of stomach muscle and two inhibit the contraction. This implies that some BLPs are the antagonist of bombesin receptors although most BLPs are agonists. This current finding will facilitate the identification of novel bombesin receptors and novel ligands of bombesin receptor. The precursor sizes of BLPs and their processing

patterns are found to vary in different families of amphibians, with implications for amphibian taxonomy and molecular phylogenetics.

Materials and methods

Collection of frog skin secretions

Adult specimens of *O. grahami* of both sexes ($n=30$; weight range 30–40 g) were collected in Yunnan Province of China. Skin secretions were obtained using the method described before [23]. The dorsal region of each frog was flushed with 0.1 M NaCl solution containing 0.01 M EDTA. 500 ml of skin secretion was collected and quickly centrifuged. The supernatant was lyophilized.

Peptide purification

Lyophilized skin secretion sample (2.0 g, total $OD_{280\ nm}$ of 600) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, with collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The contractile activity on isolated stomach strips of fractions was determined as indicated below. The protein peaks with contractile activity on isolated stomach strips were pooled (30 ml) and lyophilized. The product was resuspended in 2 ml 0.1 M phosphate buffer solution, pH 6.0, and finally purified using C_{18} reverse-phase high-performance liquid chromatography (Hypersil BDS C_{18}, 30 × 0.46 cm).

Bioassay

The isolated peptide was tested for bombesin-like activity by monitoring the contractile activity on the isolated rat stomach strip [28]. Briefly, male Wistar rats (200–250 g body weight) were killed by a neck dislocation. The segments of the isolated stomach strips were mounted isotonically, under 1.5-g load, in 20 ml organ bath containing Krebs solution at 37 °C and bubbled with 95% O_2 and 5% CO_2. The tissues were equilibrated for 45–60 min before being exposed to the sample tested. The biological signal was collected and analyzed using PcLab software package (Beijing Microsignalstar Technology Development Co. Ltd.).

Structural analysis

The purified peptides were treated with pyroglutamate amino peptidase (Sigma) using the method described [28], and then the amino acid sequence was determined by the automated Edman degradation method on an Applied Biosystems pulsed liquid-phase sequencer (Model 491). The status of the C terminus was investigated as described [29]. The purified peptide was digested with carboxypeptidase Y (Sigma) that was pretreated with phenylmethylsulfonyl fluoride to inactivate endopeptidase and amidase activities. Aliquots of the enzyme digests were taken at 1- and 2-h time intervals and subject to amino acid analysis (Hitachi 835–50 amino acid analyzer). FAB mass spectrometry was carried out to determine the molecular weight of purified peptides on an Autospec-3000 spectrometer, equipped with a high-field magnet, using glycerol:3-nitrobenzyl alcohol:dimethyl sulfoxide (1: 1: 1, v:v:v) as mix matrix. The ion gun was operated at 25 kV with a current of 1 μA, using Cs^+ as the bombarding gas.

Construction of the cDNA library

Total RNA was extracted from the skin of a single sample of *O. grahami* by TRIzol reagent (Life Technologies, Ltd.). The cDNA was synthesized using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The primers used in the first-strand synthesis were cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G, or C) and SMART II A oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

Screening of cDNA library for BLPs encoding sequences

The cDNA synthesized was used as template to screen the cDNAs encoding BLPs. Two oligonucleotide primers, S_1 5′-ttct(g/c)a(t/a)ggcaggaactgca(g/c)tcat-3′, in the sense direction, a specific primer designed according to the signal peptide sequences of BLPs from ranid frogs, and primer II A (see Construction of the cDNA library) in the antisense direction were used in PCRs. The DNA polymerase was Advantage polymerase from Clontech. The PCR conditions were 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, and 40 s at 72 °C. Finally, the PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer (Model ABI PRISM 377).

Synthesis of peptides

The following peptides were synthesized at AC Scientific (Xi An) Inc. (Xi An, China) and analyzed by HPLC and matrix-assisted laser desorption ionization-time of flight mass spectrometry. The purity of the synthesized peptide was determined to be higher than 95%. All peptides were solved in double distilled water: B1, pEREYRAPHWAIGHFM-NH_2; B2, pEREYRTPH-WAIGHFM-NH_2; B3, pERECRAPHWAIGHFM-NH_2; B4, pENTYRAPQ-WAVGHLM-NH_2; B5, pESTYRAPQWAVGHLM-NH_2; Phe^{13}-bombesin, pEQSLGNQWARGHFM-NH_2.

Acknowledgments

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