Gastrin-releasing Peptide (GRP) Is Not Mammalian Bombesin

IDENTIFICATION AND MOLECULAR CLONING OF A TRUE AMPHIBIAN GRP DISTINCT FROM AMPHIBIAN BOMBESIN IN BOMBINA ORIENTALIS

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On the basis of structural homology and similar biological activity, gastrin-releasing peptide (GRP) has been considered the mammalian equivalent of amphibian bombesin. In this paper we now show this to be incorrect. Chromatography of frog (Bombina orientalis) gut extracts demonstrated two peaks of bombesin-like immunoreactivity (BLI), one similar in size to GRP and one similar in size to amphibian bombesin. These peaks were purified by high pressure liquid chromatography then subjected to mass spectrometric analyses to determine molecular weights and amino acid sequence. Based on the amino acid sequence of the lower molecular weight BLI species, a mixed oligonucleotide probe was prepared and used to screen a B. orientalis stomach cDNA library. Sequence analysis showed that all hybridizing clones encoded a 150-amino acid protein homologous to the mammalian GRP precursor. The mass spectra of the high and low molecular weight peaks of frog gut BLI were consistent with their origin from the processing of the frog GRP (fGRP) precursor into GRP-29 and GRP-10, just like the processing of the rat GRP precursor. Sequence homology showed that the fGRP precursor is more closely related to the mammalian GRP precursors than to either the frog bombesin or frog ranatensin precursors. Northern blot analysis showed that fGRP is encoded by a mRNA of 980 bases, clearly different from the 750-base mRNA which encodes frog bombesin. Northern blot analysis and in situ hybridization showed fGRP mRNA in frog brain and stomach and bombesin mRNA in frog skin, brain, and stomach. That frogs have independent genes for both GRP and bombesin raises the possibility that mammals have an as yet uncharacterized gene encoding a true mammalian bombesin.

Bombesin, a 14-amino acid peptide, was originally isolated from the skin of the frog Bombina bombina by Anastasi et al. (1). Amphibian bombesin was quickly found to have multiple effects in mammals including induction of hypothermia, stimulation of DNA replication, and the release of many gastrointestinal hormones (2–5). Radioimmunoassay and immunohistochemistry demonstrated that bombesin-like immunoreactivity (BLI)1 was widely distributed in mammalian brain, gastrointestinal tract, and lung (6, 7). Chromatography of gut extracts showed two forms of mammalian BLI, one larger than amphibian bombesin and one smaller (4, 6). Using gastrin release as a bioassay McDonald and co-workers (8) isolated from porcine stomach a 27-amino acid peptide homologous to the carboxyl terminus of bombesin and named it gastrin-releasing peptide (GRP). GRP has since been characterized or cloned from rat, human, canine, avian, and guinea pig species (9–13). Reeve et al. (11) demonstrated that the smaller form of mammalian BLI was the carboxyl-terminal decapeptide of GRP. This peptide, called GRP-10, appears to be present in all species which also express GRP (11, 14–16). Because GRP reproduces all of bombesin's biologic effects, GRP and GRP-10 have been considered the mammalian equivalents of amphibian bombesin.

Walsh et al. (34) demonstrated that in frogs, BLI exists in two forms, a higher molecular weight form similar in size to mammalian GRP and a low molecular form closer in size to amphibian bombesin. Previously our laboratory has cloned cDNAs encoding amphibian bombesin from the skin of B. orientalis (18) and demonstrated that the bombesin mRNA was expressed not only in skin, but also in brain and stomach. The exact nature of BLI in frog brain and stomach, however, remained to be determined. In this paper we report that frog (B. orientalis) gut BLI does not derive primarily from the frog bombesin precursor, but rather like mammalian BLI, is predominantly GRP-29 and GRP-10.

MATERIALS AND METHODS

Animals and Reagents—Frogs (B. orientalis) were obtained from California Zoological Supply (Santa Ana, CA) and maintained on crickets. Enzymes were purchased from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), or Promega (Madison, WI). Isotopes were purchased from Du Pont-New England Nuclear.

1 The abbreviations used are: BLI, bombesin-like immunoreactivity; GRP, gastrin-releasing peptide; GRP-10, the carboxy-terminal decapetide of GRP; LSIMS, liquid secondary ion mass spectrometry; fGRF, frog GRF; MH+, protonated molecular ion; CID, collision-induced dissociation; CTEP, carboxy-terminal extension peptide; bp, base pair(s); SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; kb, kilobase(s).
**Frog Gut Extraction and Concentration—**Preliminary studies showed that intestine and stomach both had similar profiles of BLI. To avoid contamination of gut tissue with skin bombesin, separate instruments were used to remove heads and skin from 93 frogs, and the torso was rinsed in cool tap water. After removal, the stomachs and intestines were immersed in —70°C methanol on the next day. The guts (33 g) were boiled in 330 ml of 3% acetic acid for 5 min. After cooling, the extract was centrifuged at 15,000 × g for 1 h. The supernatant was loaded on four sets of SepPaks connected in series. The four sets of SepPaks were combined in series and rinsed with 20 ml of 0.1% trifluoroacetic acid. They were then eluted with 12 ml of 60% acetonitrile containing 0.1% trifluoroacetic acid.

**Chromatography of Frog Extracts—**The 12 ml of SepPak eluate was diluted to 50 ml and loaded onto a Sephadex G-50 (5 × 95 cm) column equilibrated in 3% acetic acid. The column was eluted with the same buffer and 8-ml fractions were collected. The fractions were monitored for absorbance at 280 nm and BLI. The early and late eluting BLI from the gel permeation chromatography were pooled and loaded separately onto a Preparative Ramin C-18 column (2.2 × 25 cm). The column was rinsed with 0.1% trifluoroacetic acid then eluted with a 5-min gradient to 10% buffer B (60% acetonitrile containing 0.1% trifluoroacetic acid), followed by a 110-min linear gradient to 75% buffer B. The flow rate was 5 ml/min; 1-ml fractions were collected, the effluent was monitored at 220 nm, and the fractions were assayed for BLI. Fractions containing BLI were pooled, diluted 3-fold, and loaded onto an analytical (4.9 mm × 25 cm) Vydac C-18 column. The column was rinsed, then eluted with a 5-min gradient to 35% B, followed by a 60-min gradient to 65% B. The flow rate was 1 ml/min, 1-ml fractions were collected, the effluent was monitored at 220 nm, and the fractions were assayed for BLI. The C-4 purified material was then subjected to mass spectrometric analysis as described below.

**Radioimmunoassay of Bombesin-like Immunoreactivity—**Bombesin-like immunoreactivity was measured by a radioimmunoassay procedure described previously (11). The antiserum (1078) used is specific for the carboxyl-terminal portion of bombesin and gastrin-releasing peptide which are identical in 9 of 10 residues, and its affinity for amphibian bombesin and mammalian GRP are nearly identical.

**Mass Spectrometry—**Peptide fractions containing BLI were first analyzed by liquid secondary ion mass spectrometry (LSIMS). Briefly, aliquots of the samples were dissolved in water, dried on the probe, and approximately 1 μl of glycerol/dithioerycroglycerol (1:2) was added. For analysis in the low mass range (<2000 Da) a Kratos MS-505 mass spectrometer equipped with a high field magnet and postacceleration detector was used as previously described (19). A Cs+ primary beam energy of 10 keV was used to generate the secondary sample ions, which were accelerated at 8 keV. Scans were taken at either 100 or 500 s decade, recorded on a Gould electrostatic recorder and calibrated manually with a Ultramark external reference to an accuracy better than ±0.2 Da. Alternatively, a Kratos Concept HH tandem mass spectrometer (20) equipped with a LSIMS source and postacceleration detector and/or a VG Bio-Q quadrupole mass spectrometer equipped with an electrospray source (21) were used for high mass analyses (>2000 Da). For the LSIMS analyses, seven consecutive spectra were taken over the mass range 5000–1500 at a scan rate of 10 s/decade and averaged. Mass assignment was carried out automatically via a Mach 3 data system using a Geli-Calibrant. Electro-spray spectra were acquired on the VG Bio-Q instrument by injecting a 10-μl aliquot via an ABI 140A syringe pump into a steady stream of acetonitrile/H2O (1/1, by volume) at a flow rate of 5 μl/min. The quadrupole was scanned from m/z 500–2000 in 10 s and eight scans were averaged. Calibration was carried out automatically using the VG Bio-Q software package and horse heart myoglobin as the external reference.

To sequence the peptides identified in the LSIMS survey scans, a Kratos Concept tandem mass spectrometer operating in a constant B/E mode under collision-induced dissociation (CID) conditions was used (22). Peptides were prepared using a glycerol/dithioerycroglycerol matrix and infused into a Michelson Amino acid sequencer (23) with an Oxford1200 source as described above. In each case, the C-12 containing isolate was selected from the parent protonated molecular ion (MH+) in MS1 and passed through a collision cell floated at 2 kV containing sufficient helium to reduce the parent ion abundance to one-third of its initial value. The resulting fragment ions were then separated from the recombined ions using a composite ion scan and recorded in 4% mass intervals on a 1-inch diode array detector. Mass assignments were made using a Mach3 data system and Cal as the external calibrant. Spectra were interpreting with the aid of a previously described computer algorithm (22).

**Oxidation of Peptide Fractions—**In order to identify peptides containing methionine and/or tryptophan for further structural characterization, BLI-containing fractions were oxidized according to the procedure of Wagner and Frazer (23). In this procedure, methionine is oxidized to methionine sulfone and tryptophan to oxyindolalanine, resulting in the addition of 18 kDa to the molecular mass of each of these amino acid present. Briefly, a 100-μl aliquot containing approximately 50 pmol of the low molecular weight BLI was transferred to a 0.5-ml polypropylene tube and dried down in a Speed-Vac concentrator (Savant Instruments). 5 μl of the oxidizing reagent (redistilled dimethyl sulfoxide, 9 N HCl, acetic acid, 4/15/30 by volume) was added and incubated at room temperature for 30 min. The peptide was then dried under vacuum without further purification by LSIMS as described above. As positive controls, both synthetic bombesin (Peninsula Labs, La Jolla, CA) and substance P (Sigma) in 1-nmol amounts were subjected to the identical derivatization procedure.

**cdNA Library Construction and Screening—**Total and poly(A)+ RNA were prepared by standard methods (24) using guanidine thiocyanate and oligo(dt) from 20 pooled stomachs of B. orientalis. A cdNA library was prepared in the vector λ ZapII using reagents and protocols supplied by Stratagene (Unzip) and packaged into lambda phage using Gigapack Gold (Stratagene). Total library size was 1.5 × 1010 with an average insert size of 1 kb. Based on the BLI amino acid sequence derived from the mass spectrometric analysis, the library was screened with a 32P 5′-end-labeled mixed oligonucleotide probe corresponding to the following sequence: GGGTGGGCGCGAGATGGTG(Tg)GC; H = A, C, or T, Y = C or T; and I = deoxyinosine). For hybridization, nitrocellulose filters were prehybridized at 37°C for 16 h in 6 × SSC/5 × Denhard’s/0.1% SDS/200 μg/ml sonicated denatured salmon sperm. 32P-Labeled probe was added and the filters hybridized at 40°C for another 24 h. Filters were washed four times for 15 min at 42°C in 6 × SSC/0.1% SDS. Hybridizing phage were preclarified and extensively washed with the phosphate buffer following the supplier’s (Stratagene) protocol. Sequence analysis was performed on double-stranded template, initially using flanking vector sequence as primers and then by using internal sequences as primers. All sequence was obtained on two independent clones and on both strands. Oligonucleotides were synthesized on an ABI model 391 DNA synthesizer. Database analysis and sequence alignment was done with the DNASTAR software (Madison, WI) or GCG version 7.0 (Madison, WI) (25).

**RNA Blot Analysis—**Total and poly(A)+ RNA were isolated as described above. RNA was separated on 1.5% formaldehyde-agarose gels, transferred to nylon membrane by capillary transfer, and UV cross-linked. For hybridization, 32P-labeled antisense cRNA was transcribed from plasmids encoding frog bombesin (18) or frog GRP. Hybridization conditions were 65°C formamide, 5 × SSC, 5 × Denhard’s, 100 μg/ml sonicated denatured salmon sperm, 200 μg of yeast RNA at 65°C for 18 h. Blots were washed for two 15-min intervals in 2 × SSC, 0.1% SDS at 22°C followed by two 15-min intervals in 0.1 × SSC, 0.1% SDS at 65°C.

**In Situ Hybridization Analysis—**Procedures were modified after Cox et al. (26) and Smirner and Young (27). In brief, B. orientalis frogs were injected with 0.5 ml of 1 μM norepinephrine in order to boost skin levels of bombesin (28) and maintained for 2 weeks. Frogs were anesthetized with 0.1% Lidocaine™ and perfused through the heart with 4% paraformaldehyde in borate buffer (pH 9.5) for 10 min. Skin, stomach, and brain were dissected and postfixed in 4% paraformaldehyde in borate buffer containing 0.1% sucrose for 24 h, and 15-μm sections were cut on a cryostat. 35S Labeled antisense bombesin cRNA and frog GRP cRNA probes were prepared by in vitro transcription as described above. Mounted sections were treated with proteinase K 10 μg/ml for 30 min, acetylated, dehydrated, and hybridized with 2.5–5 × 106cpm/ml of probe at 60°C overnight. Slides were treated with RNase A (20 μg/ml) for 30 min at 37°C and then washed at decreasing concentrations from 2 × SSC to 0.5 × SSC at room temperature with a final wash at 65°C in 0.1 × SSC for 30 min. The slides were exposed to Beta Max film (Amersham Corp.) for 24 h and then dipped in NTB3 liquid emulsion (Kodak, Rochester, NY) and exposed for 7–14 days. Slides were counterstained with hematoxylin-eosin prior to examination.
RESULTS

Frog Gut Extraction and Chromatography—Frog gut tissue contained approximately 150 pmol/g of BLI. Chromatography on Sephadex G-50 revealed two peaks of BLI (Fig. 1), one eluting similarly to porcine GRP-27 (peak 1) and one eluting similarly to frog bombesin-14 (peak 2).

The two peaks from the Sephadex G-50 column (Fig. 1) were pooled and chromatographed separately on a preparative C-18 HPLC column. The earlier eluting G-50 peak (peak 1, Fig. 1) eluted as a single immunoreactive peak which did not correspond with a unique absorbance peak (Fig. 2A). The later eluting G-50 peak (peak 2, Fig. 1) eluted as one major and one minor peak (Fig. 2B). The major peaks from these columns were purified through another step of reversed-phase HPLC, but no unique absorbance was associated with the immunoreactive peak (data not shown). However, the tubes containing the highest concentration of immunoreactivity were believed pure enough for mass spectral analysis.

Mass Spectrometry—Analysis of low molecular weight BLI (peak 2, Fig. 1). The LSIMS spectrum of this fraction showed a number of protonated molecular ions ranging in mass from MH⁺ 901 to MH⁺ 1558. To identify which derived from the bombesin-like peptide, the total fraction was oxidized under conditions where the conserved methionine residue would be converted to methionine sulfoxide. Thus molecular ions that shift by n × 16 Da would likely derive from reduced bombesin-like peptides. LSIMS analysis before and after such treatment showed a mass shift in only one of the molecular ion components (Fig. 3). In these spectra (only partial spectra are shown) the MH⁺ ion at m/z 1093 of the untreated fraction is virtually absent in the oxidized fraction, and two new peaks appear at m/z MH⁺ 1109 (+16 Da) and MH⁺ 1125 (+32 Da). This suggests the presence of two oxidizable residues, and is consistent with the presence of one methionine and one tryptophan typical of most bombesin-like peptides.

For this component, tandem mass spectrum was obtained from its molecular ion for sequence analysis. Shown in Fig. 4 is the CID spectrum of the m/z 1093 ion of the nonoxidized fraction. Computer-aided sequence analysis established the identities of the carboxyl-terminal 7 amino acids as -Trp-Ala-Val-Gly-His-Leu-Met-amide (22). The precise identity of the amino-terminal region was ambiguous. Subsequent manual interpretation assigned the sequence as Gly-Ser-His-Trp-Ala-Val-Gly-His-Leu-Met-amide based on the presence of several internal dipeptide ions including those for Ser-His at m/z 197 (y7b3) and 225 (y8b3) and for His-Trp at m/z 296 (y7b3) and 324 (y9b3).

Isolation and Analysis of cDNA Encoding for Frog grp—

**Fig. 1.** Chromatography of crude frog gut extracts. Tissue extracts were fractionated on a 1 × 95-cm Sephadex G-50 superfine column. Fractions were collected and assayed for BLI as described under “Materials and Methods.” Elution position of synthetic porcine GRP-27 and amphibian bombesin-14 (BBS) are as shown.

**Fig. 2.** HPLC purification of frog gut BLI. A, purification of higher molecular weight BLI (peak 1, Fig. 1). B, purification of lower molecular weight BLI (peak 2, Fig. 1). HPLC conditions as described under “Materials and Methods.”

**Fig. 3.** Partial LSIMS spectra of low molecular weight BLI peak (A) (peak 2, Fig. 1) before and (B) after oxidative treatment showing the shift in the MH⁺ ion specie at m/z 1093 to m/z 1109 (+16, O) and 1125 (+32, O₂).
Based on the amino acid sequence predicted by mass spectrometry, a mixed oligonucleotide was synthesized and used to screen a *B. orientalis* stomach library as described under "Materials and Methods." Two hybridizing clones were isolated. All clones showed a similar-sized cDNA insert of 1 kb, and two such clones were sequenced in their entirety on both strands. Sequence analysis revealed an open reading frame of 453 nucleotides encoding a 155-amino acid protein (Fig. 5).

The cDNA contained 172-bp 5'-untranslated and 339-bp 3'-untranslated regions, and a polyadenylation signal (AATAAA) was located at position 941 followed by a poly(A) tail. The open reading frame encoded a hydrophobic signal peptide, a dibasic (Lys-Lys) cleavage signal at position 127, and then a carboxy-terminal extension peptide (CTEP). The CTEP contained a second dibasic (Lys-Lys) cleavage signal and a carboxy-terminal methioninamid of GRP.
mone amino acid sequence (Fig. 5) and the LSIMS spectra of the higher molecular weight BLI fraction (Fig. 6), the signal peptide cleavage point and the sequence of frog GRP could be deduced. The LSIMS of this fraction yielded a series of MH\textsuperscript{+} ions as shown in Fig. 6. The largest of these had a m/z of 3174.5 (exact mass for the C-12 containing molecular ion component) (see inset, Fig. 6). Based on the cDNA sequence of the Bombina GRP precursor, this matches the predicted monoisotopic C-12 mass of the peptide fragment starting from Ser-32 and ending at Met-amine-61 (MH\textsuperscript{+} 3174.6 calculated).

Data Base Analysis—The amino acid sequence of fGRP and its prohormone were compared to that of bombesin, ranatensin, and the other known GRPs (Fig. 7). Homology was higher between the fGRP prohormone and the human or rat GRP prohormones (homology scores, 191–199) than between the fGRP and bombesin prohormones (homology score, 97). This indicates that the bombesin genes separated from the GRP genes evolutionarily quite distantly. By contrast, bombesin showed highest homology with ranatensin with a homology score of 196. This suggests that a primordial gene duplicated to give rise to a bombesin-like peptide and a GRP-like peptide and then the bombesin gene duplicated to give rise to a ranatensin-like peptide and a bombesin-like peptide.

Northern Blot Analysis—The expression of fGRP mRNA in skin, gut, and brain of B. orientalis was compared to the expression of the bombesin mRNA (Fig. 8). The 750-base bombesin mRNA was present in skin, stomach, and brain. Bombesin mRNA expression was maximal in skin (skin > brain > stomach). Highest levels of the 980-base fGRP mRNA were in stomach. No fGRP mRNA was detected in skin even probing high amounts of poly(A) RNA. Low levels of GRP mRNA were detected in brain, although the brain mRNA appeared slightly larger in size (1100 bp, data not shown) than the stomach GRP mRNA. In stomach, GRP mRNA was more abundant than bombesin mRNA; in brain, bombesin mRNA was more abundant than GRP mRNA.

In Situ Hybridization Analyses of Bombesin and GRP mRNAs—In situ hybridization analysis using \textsuperscript{35}S-cRNA probes was done to more precisely study the distribution of bombesin and GRP in the skin and stomach (Fig. 9). In the skin, bombesin mRNA was localized in the cutaneous granular glands consistent with previous immunohistochemical studies of frog skin peptides (29, 30). In the stomach, GRP mRNA was localized to occasional cells at the base of the gastric pits. The distribution and appearance of these cells were consistent with that of gut neuroendocrine cells (31, 32) and was similar to that reported for bombesin-like immunoreactivity by Lechago et al. (31, 32). Though low levels of bombesin mRNA could be detected in frog stomach poly(A) RNA by Northern blot, no clearly labeled cells were detected by in situ hybridization.

**Discussion**

Bombesin was originally characterized from the skin of Bombina bombina (1) and has also been demonstrated in the skin of B. orientalis (33). Chromatography of tissue extracts from several frog species, including Rana (34), Xenopus (34), and Bombina (Fig. 1), demonstrate two forms of BLI in gut and brain; a higher molecular weight species consistent with GRP and a smaller species closer in size to bombesin. The exact nature of this BLI has heretofore been unknown. The two peaks of B. orientalis gut BLI were purified by gel filtration and HPLC, and then subjected to analysis by mass spectrometry. From the derived amino acid sequence, oligonucleotide probes were synthesized to allow the cloning of cDNAs encoding frog gut BLI. Sequence analyses of these cDNAs showed that frog gut BLI is similar to mammalian GRP and GRP-10.

The structure of the fGRP prohormone (Fig. 5) is similar to that of the rat and human GRP prohormones (10, 35). There is a 31-amino acid signal peptide followed by the 29-amino acid GRP peptide, a dibasic amino acid cleavage site, and a 92-amino acid CTEP. Within the CTEP a second dibasic cleavage site is present that could generate other functional peptides. This is remarkably similar to the rat in which a 29-amino acid GRP is also followed by a 92-amino acid GRP prohormone (10).

Frog GRP was shown to be 29 amino acids in length by determination of the molecular mass of the major species in the high molecular weight BLI fraction (Figs. 1 and 6). This implies that GRP-29 is cleaved from the signal peptide after Ser-Glu-Ala, which is consistent with the signal peptidase recognition site consensus (36) and is similar to the cleavage points for human and rat GRP (Gly-Arg-Ala and Gly-Pro-Ala, respectively). While human, porcine, avian, guinea pig, and canine GRP are all 27 amino acids (8, 11-13, 15), rat GRP has been shown to be 29 amino acids by molecular cloning (35) and amino acid sequencing.

The predicted sequence for the low molecular weight frog gut BLI species is identical to rat and chicken GRP-10. In all species examined to date GRP exists in two forms, a full size 25–29-amino acid peptide and the carboxy-terminal decapeptide of GRP, called GRP-10. The sequence of GRP-10 is identical in all species except for position 2 which is Ser in chickens, rats, and frogs and Asn in pigs, dogs, and humans.

Alignment of prohormone sequences (Fig. 7) showed frog GRP has highest homology with rat GRP and human GRP. The homology to frog bombesin and frog ranatensin was substantially lower. Homology between the frog, rat, and human GRP prohormones showed surprisingly strong conservation in the CTEPs (Fig. 7). There are three forms of the human GRP prohormone (type I, II, and III) which identically encode GRP but encode different CTEPs (37, 38). The frog GRP CTEP is more closely related to the human type I CTEP.

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\(2\) J. Reeve, unpublished data.

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**Fig. 6.** Partial LSIMS of the high molecular weight BLI species peak (peak 1, Fig. 2), showing the four major MH\textsuperscript{+} components (reported as the exact masses of their C-12 containing isotopic masses). The expanded mass region for the MH\textsuperscript{+} ion at m/z 3174.5 is shown in the top right.
Fig. 7. Comparisons of fGRP and its prohormone with related sequences. A, alignment of the fGRP and amphibian bombesin precursors (33). B, alignment of the fGRP and human GHP type 1 precursors (32, 38). C, optimized alignment scores obtained after aligning the fGRP precursor with the precursors for bombesin, ranatensin (41), rat GRP (35), human GRP type 1 and 2, and also alignment of the ranatensin precursor to that of bombesin and neuromedin B (41). D, alignment of mature GRP peptides from frog, dogfish (42), chicken (12), rat (35), pig (8), dog (11), and human (10, 15). Residues identical to those of fGRP are boxed. M*, methioninamide; pQ, pyroglutamate. Single alignments for A–C were performed using software provided by DNASTAR (AALIGN, version 1.68). Multiple alignment for D was performed with PileUp, GCG version 7.0 (25).

Fig. 8. Northern blot analysis of bombesin and GRP mRNA expression in B. orientalis. Total or poly(A)+ RNA was resolved on 1.5% formaldehyde-agarose, transferred to a nylon membrane, and probed with the bombesin (lanes 1–3) or GRP cRNA (lanes 4 and 5) probes as described under “Materials and Methods.” Lane 1, 2.5 µg of total RNA from norepinephrine-stimulated dorsal skin (sk); lanes 2 and 5, 10 µg of poly(A)+ RNA from stomach (st); lane 3, 10 µg of total RNA from brain (br); lane 4, 10 µg of poly(A)+ RNA from norepinephrine-stimulated dorsal skin. Because of the large differences in RNA levels, the same autoradiogram was exposed for three different times. Lane 1 shows a 15-min exposure; lanes 2, 3, and 5 show a 24-h exposure, and lane 4 shows a 72-h exposure.
FIG. 9. In situ hybridization analysis of the expression of bombesin and GRP mRNAs in B. orientalis skin. A. skin probed with 35S-bombesin cRNA probe (250x); arrows show intense hybridization in cells lining the cutaneous granular gland. B and C were probed with 35S-GRP cRNA probe. B shows skin section adjacent to A (100 x) with no apparent hybridization. Arrows point to glands which lack hybridization. C shows cells at the bottom of the gastric pit (400x) hybridizing to the 35S-GRP probe. Arrows point to the hybridizing cells.

with the recent report by Conlon et al. (39) who determined the protein sequence of a bombesin-like peptide in the brain of Rana ridibunda and found it to be identical to the sequence of fGRP-10 we report here. In situ hybridization localized bombesin mRNA to the cutaneous granular glands in skin and localized GRP mRNA to apparent neuroendocrine cells in frog stomach. No bombesin mRNA containing cells in stomach were localized by in situ hybridization. This is consistent with the lower levels of bombesin mRNA found in stomach by Northern blot (Fig. 8) and suggests a very limited distribution of bombesin cells in gut, perhaps a subset of gut neurons. While levels of bombesin mRNA were quite low in stomach, bombesin mRNA was easily detected in brain. That both bombesin and GRP are expressed in frog brain and gut argues that both may have functions as neurotransmitters or neuromodulators. If both bombesin and GRP act as neurotransmitters in frogs, then both GRP and bombesin may also act as neurotransmitters in mammals. This further argues for the existence of a true mammalian bombesin, and from the sequence comparisons we speculate that the family of mammalian bombesin-like peptides and its receptors may be surprisingly large. Studies to identify such peptides are currently underway.

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