NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

Molecular Cloning of the Helodermin and Exendin-4 cDNAs in the Lizard: RELATIONSHIP TO VASOACTIVE INTESTINAL POLYPEPTIDE/PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE AND GLUCAGON-LIKE PEPTIDE 1 AND EVIDENCE AGAINST THE EXISTENCE OF MAMMALIAN HOMOLOGUES

Markus Pohl and Stephen A. Wank

J. Biol. Chem. 1998, 273:9778-9784.
doi: 10.1074/jbc.273.16.9778

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 16, Issue of April 17, pp. 9778–9784, 1998
Printed in U.S.A.

Molecular Cloning of the Helodermin and Exendin-4 cDNAs in the Lizard

RELATIONSHIP TO VASOACTIVE INTESTINAL POLYPEPTIDE/PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE AND GLUCAGON-LIKE PEPTIDE 1 AND EVIDENCE AGAINST THE EXISTENCE OF MAMMALIAN HOMOLOGUES*

(Received for publication, September 3, 1997)

Markus Pohl and Stephen A. Wank‡

From the Digestive Diseases Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

Helodermin and exendin-4, two peptides isolated from the salivary gland of the Gila monster, *Heloderma suspectum*, are approximately 50% homologous to vasoactive intestinal peptide (VIP) and glucagon-like peptide-1 (GLP-1), respectively, and interact with the mammalian receptors for VIP and GLP-1 with equal or higher affinity and efficacy. Immunohistochemical studies suggested the presence of helodermin-like peptides in mammals. To determine whether helodermin and exendin-4 are present in mammals and their evolutionary relationship to VIP and GLP-1, their cDNAs were first cloned from Gila monster salivary gland. Northern blots and reverse transcription-polymerase chain reaction of multiple Gila monster tissues identified ~500-base pair transcripts only from salivary gland. Both helodermin and exendin-4 full-length cDNAs were ~500 base pairs long, and they encoded precursor proteins containing the entire amino acid sequence of helodermin and exendin-4, as well as a 44- or 45-amino acid N-terminal extension peptide, respectively, having ~60% homology. The size and structural organization of these cDNAs indicated that they were closely related to one another but markedly different from known cDNAs for the VIP/GLP-1 peptide family previously identified in both lower and higher evolved species. Cloning of the Gila monster VIP/peptide histidine isoleucine, pituitary adenylate cyclase activating polypeptide, and glucagon/GLP-1 cDNAs and Southern blotting of Gila monster DNA demonstrate the coexistence of separate genes for these peptides and suggests, along with the restricted salivary gland expression, that helodermin and exendin-4 coevolved to serve a separate specialized function. Probing of a variety of rat and human tissues on Northern blots, human and rat Southern blots, and genomic and cDNA libraries with either helodermin- or exendin-4-specific cDNAs failed to identify evidence for mammalian homologues. These data indicate that helodermin and exendin-4 are not the precursors to VIP and GLP-1 and that they belong to a separate peptide family encoded by separate genes. Furthermore, the existence of as yet undiscovered mammalian homologues to helodermin and exendin-4 seems unlikely.

A large number of nonmammalian peptides that are bioactive in mammals, and thus are able to interact with mammalian receptors, have been discovered in species such as amphibians, reptiles, and insects. In particular, frog skin and reptile and insect venoms are rich sources of such bioactive peptides (2–5). Although mammalian homologues for some of these peptides have been demonstrated (6, 7), others have been suspected for some time on the basis of immunohistochemical studies and the bioactivity of these peptides in mammals (3). The difficult and often laborious process of isolating and sequencing the mammalian homologues in some instances has contributed to the delay in their discovery. Recent advances in molecular biological methods have provided sensitive and specific tools for speeding this discovery process and also have the potential to provide insight on genetic evolutionary relationships among these peptides that are present in different species (8–11).

In 1982, it was observed that the crude venom of the Gila monster (*Heloderma suspectum*) was a potent pancreatic secretagogue (12). Purification and sequencing of the active factors mediating this effect led to the discovery of the peptides helodermin and exendin-4 (13, 14). Helodermin, a peptide consisting of 35 amino acids, shares 53 and 42% homology with human pituitary adenylate cyclase activating peptide (PACAP)[1] and vasoactive intestinal peptide (VIP), respectively (Fig. 1). It was shown to have high affinity for the mammalian VIP_2 receptor and equal potency and efficacy for stimulating cAMP production compared with mammalian VIP and PACAP (15). In some studies, even a helodermin-preferring receptor has been described (16, 17). Furthermore, immunohistochemical studies, using antibodies that did not cross-react with mammalian VIP or PACAP or other known members of the glucagon-like peptide-1 (GLP-1)/VIP/PACAP peptide family suggested the existence of a mammalian homologue to helodermin distinct from VIP and PACAP (18–21). Exendin-4, a 39-amino acid peptide, is most homologous (53%) to mammalian GLP-1 (Fig. 1). It was shown to bind with high affinity to the mammalian GLP-1 receptor and to exhibit nearly equal potency and efficacy for stimulating cAMP production compared with mammalian GLP-1-(7–36) (22). Furthermore, exendin-4 and GLP-1-(7–36) were found to exhibit similar potencies and efficacies in stimulating other biological responses that are believed to be mediated by the GLP-1 receptor, such as acid secretion from parietal cells and insulin secretion from β cells in pancreatic

* During the preparation of this paper, the cloning of the exendin-4 cDNA from *H. suspectum* was published (1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Bldg. 10, Rm. 9C-103, National Institutes of Health, Bethesda, MD 20892-1804, Tel.: 301-496-4201; Fax: 301-402-0600.

[1] The abbreviations used are: PACAP, pituitary adenylate cyclase activating polypeptide; VIP, vasoactive intestinal polypeptide; GLP-1, glucagon-like peptide 1; PHI, peptide histidine isoleucine; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends.

```
HELODERMIN:   HSDAIFTEEYSKLLAKLALQKYLASILGSRTSPPP*
HUMAN PACAP:  HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK*
HUMAN VIP:    HSDAVFTDNYTRLRKQMAVKKYLNSILN*

EXENDIN-4:          HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS*
HUMAN GLP-1(7-36):  HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR*
HUMAN GLUCAGON:     HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
```

FIG. 1. **Alignment of the peptide sequences of helodermin to human PACAP and VIP (*top*) and exendin-4 to human GLP-1 and glucagon (*bottom*).** Conserved amino acid residues that are identical are shown in *boldface*. Peptide C-terminal α-amidation is indicated by a *star*.

islets (23, 24). The existence of a specific exendin receptor on guinea pig pancreatic acini was suggested (3). The truncated exendin peptide (9–39) is a potent antagonist of the mammalian GLP-1 receptor (22–24). Based on these findings, there has been speculation that helodermin and exendin-4 are the evolutionary precursors to the known mammalian peptides PACAP or VIP and GLP-1, respectively, as well as speculation about the existence of as yet unknown mammalian homologues.

Therefore, this study was designed to search for mammalian homologues to the reptilian peptides helodermin and exendin-4 and to examine the evolutionary relationship between helodermin and mammalian PACAP and VIP on the one hand and exendin-4 and mammalian GLP-1 on the other hand. The cloning of the helodermin and exendin-4 cDNAs revealed that the two reptilian peptides are, despite their differences in biological activity, evolutionarily closely related. They are, however, evolutionarily not related to mammalian PACAP/VIP or GLP-1, with which they share biological activities. Helodermin and exendin-4 seem to have a highly specialized function in the Gila monster as components of the venom because they do not seem to be expressed in tissues outside the salivary gland. Although we were able to identify Gila monster PACAP, VIP, and GLP-1, our extensive search for mammalian homologues to helodermin and exendin-4 was unsuccessful.

EXPERIMENTAL PROCEDURES

Materials—A Gila monster (*H. suspectum*) was obtained from the Adobe Mountain Wildlife Center, Arizona Game and Fish Department (Phoenix, AZ). Denhardt's solution (100×) and sheared salmon sperm DNA (10 mg/ml) were from 5 Prime → 3 Prime, Inc. (Boulder, CO). 1 M Tris (pH 7.4), 20× SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0), and SDS were purchased from Digene Diagnostics, Inc. (Beltsville, MD). Formamide, guanidine isothiocyanate, and formaldehyde solution were from Fluka Chemical Corp. (Ronkonkoma, NY). Guanidine Hydrochloride was from ICN Biomedicals, Inc. (Aurora, OH). All restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA).

Construction and Screening of a cDNA Library from Gila Monster Salivary Gland—Total RNA was isolated from the salivary gland using the guanidine isothiocyanate/guanidine hydrochloride method (25). Poly(A)$^+$ RNA was purified using the Poly(A) Pure^{TM} mRNA Isolation Kit (Ambion Inc., Austin, TX) and reverse transcribed using the Superscript^{TM} Preamplification System (Life Technologies, Inc.). A cDNA library was constructed using the Lambda ZAP II cloning vector (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Approximately 750×10^3 plaques were lifted onto duplicate nitrocellulose filters (Schleicher & Schuell) and hybridized under low stringency conditions (10× Denhardt's solution, 2× SSC, and 200 mg/ml denatured salmon sperm DNA) at 37 °C with either helodermin or exendin-4 ^{32}P end-labeled degenerate oligonucleotide probes derived from their respective primary amino acid sequences. The probes were labeled to a specific activity of approximately 3×10^9 cpm/mg and added to the hybridization buffer to a concentration of 1×10^6 cpm/ml. The sequence of the helodermin probe was GATGCIATITT(T/C)ACIGA (A/G)GA(A/G)TAC (I = inosine), corresponding to the amino acid sequence DAIFTEEY. The sequence of the exendin probe was AAICA(A/G)ATGGAIGA(A/G) GA(A/G)GCIGT, corresponding to the amino acid sequence KQMEEEAV. After hybridization, filters were washed twice under low stringency conditions for 15 min at room temperature with 2× SSC, 0.1% SDS and exposed to Kodak X-OMAT (XAR2) film for 48 h at −80 °C with two intensifying screens. Positive clones were plaque purified and sequenced using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and an automated 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Northern Blot Analysis—Poly(A)$^+$ RNA (0.05 μg/lane) isolated from the Gila monster salivary gland as described above was size fractionated through a 1.5% agarose denaturing formaldehyde gel and blotted overnight onto a nitrocellulose membrane (Bio-Rad). Following a 2-h prehybridization period at 50 °C, the membrane was hybridized (5× Denhardt's solution, 40% formamide, 4× SSC, 0.5% SDS, 20 mM Tris, and 100 mg/ml denatured salmon sperm DNA) to helodermin or exendin-4-specific cDNA probes for 14 h at 50 °C. The probes were generated by PCR using cDNA-specific primers. The helodermin probe was 265 bp long and corresponded to nucleotides 115–379 in Fig. 2. The exendin-4 probe was 263 bp long and corresponded to nucleotides 152–414 in Fig. 3. The probes were ^{32}P random prime-labeled to a specific activity of approximately 3×10^9 cpm/mg and added to the hybridization solution to a concentration of 1×10^6 cpm/ml. After hybridization, the membranes were washed twice under high stringency conditions for 15 min with 0.1× SSC, 0.1% SDS. The blot was analyzed using a phosphorimager, the image analysis software MacBAS, and the Fujix Pictography 3000 digital image printer (Fuji Photo Film U. S. A., Inc., Elmsford NY). The size of the hybridizing transcripts were determined using Century RNA size markers from Ambion.

RT-PCR Analysis of Gila Monster Tissues—Total RNA from Gila monster pancreas was prepared using the low temperature guanidine isothiocyanate/guanidine hydrochloride method (25). Total RNA from all other Gila monster tissues was prepared using the Total RNA isolation kit from Ambion. Poly(A)$^+$ RNA from Gila monster salivary gland was purified as described above. RT-PCR was performed on 0.05 μg of poly(A)$^+$ RNA from the salivary gland and 5 μg of total RNA from all other tissues using the Superscript^{TM} Preamplification System (Life Technologies, Inc.). PCR amplification of the first strand cDNA (35 cycles; annealing temperature, 50 °C; extension temperature, 72 °C) was performed using the following helodermin or exendin-4 cDNA-specific primers: 5′-TTATCTTCTGAAGACTCAGAAAC-3′ (helodermin sense), 5′-TAATTGGAAACAAAGACATGT-3′ (helodermin antisense), 5′-CTGCAAGCTCAGAAAGCTTTGC-3′ (exendin-4 sense), and 5′-GCTCACTAATTGGAAACAAAGAC-3′ (exendin-4 antisense). The expected 265-bp helodermin and 263-bp exendin-4 PCR products were analyzed on a 1.2% agarose gel.

PCR Cloning of Partial cDNA Sequences Encoding Gila Monster Peptides—Total RNA from Gila monster brain, pancreas, and lung was isolated and reverse transcribed to first strand cDNA as described above for RT-PCR analysis. Partial cDNA sequences encoding the full or partial peptide sequence of Gila monster PACAP, peptide histidine isoleucine amide (PHI)/VIP, and glucagon/GLP-1 were cloned by PCR amplification of the first strand cDNA from brain, lung, and pancreas, respectively. Degenerate primers were derived from either the amino acid sequences or their respective nucleotide sequences conserved among the peptides and their cDNAs in multiple species (26–35). For PACAP, PCR (35 cycles; annealing temperature, 50 °C) with the primers 5′-CCA(C/T)TCGGA(C/T)GG(G/C)AT(C/T)TTCAC-3′ (sense) and 5′-(C/G)CGTCCTTTGTT(C/T)(C/T)TA(A/T)(A/C)(C/T)CT-3′ (antisense) resulted in a 122-bp product encoding the midsection of the peptide. For glucagon and GLP-1, PCR (30 cycles; annealing temperature, 55 °C) with the primers 5′-GG(A/C)ACITTC(A/T)C(C/T)A(A/GIGACTA(C/TAG(C/T)AA-3′ (sense) and 5′-CCAIG(A/C)AA(C/T)(A/G)AA(G/T)TC(C/T)TT(G/T)GCIGC-3′ (antisense) yielded a 255-bp product encoding the 17 C-terminal amino acids of glucagon, a 28-amino acid intervening peptide, and the 23 N-terminal amino acids of GLP-1. For PHI, PCR (35 cycles; annealing temperature, 50 °C) with the primers 5′-CGIAA(C/T)GCI(A/C)GXCA(C/T)GCIGA(C/T)GG-3′ (sense) (*X* = GATC) and 5′-TAGTT(A/G)TCIGT(A/G)AAXAGIGC(A/G)TC-3′ (antisense) amplified a 182-bp product encoding the 23 C-terminal amino acids of PHI, a 20-amino acid intervening peptide, and the first two N-terminal amino acids of VIP. The cDNA encoding the C-terminal amino acids of VIP was cloned using a 3′-RACE strategy. 5 μg of total RNA from Gila monster lung were reverse transcribed to first strand cDNA with the oligo(dT) 3′-RACE primer 5′-ACTCTAGAGTGTAGACATGCATCTCACTTTAG-TAGAACTGG(T_{18})-3′, designed to contain the two separate primers 5′-ATCTCACTTTAGTAGAACTGG-3′ (RACE primer 1) and 5′-ACTCTAGAGTGTAGACATGC-3′ (RACE primer 2) for nested PCR. PCR (25 cycles; annealing temperature, 59 °C) with the sense primer 5′-GTGCTGGGCCAGTTATCAG-3′ derived from the Gila monster PHI sequence and the antisense RACE primer 1, followed by nested PCR (25 cycles; annealing temperature, 59 °C) with the nested sense primer 5′-TATCTGCATTCTCTGATGGC-3′ derived from the Gila monster PHI sequence and the antisense RACE primer 2 resulted in a candidate PCR product of approximately 430 bp. A second nested PCR (20 cycles;

FIG. 2. **Helodermin cDNA and predicted amino acid sequence.** Shown is the nucleotide sequence of the entire cloned helodermin cDNA and the deduced amino acid sequence of the helodermin precursor protein comprising the mature helodermin peptide and a 44-amino acid N-terminal extension peptide. The sequence of the mature helodermin peptide, as demonstrated previously by peptide sequencing, is *boxed*. The dibasic cleavage site of the precursor protein is indicated by an *arrow*, and the putative mRNA polyadenylation signal is *underlined*.

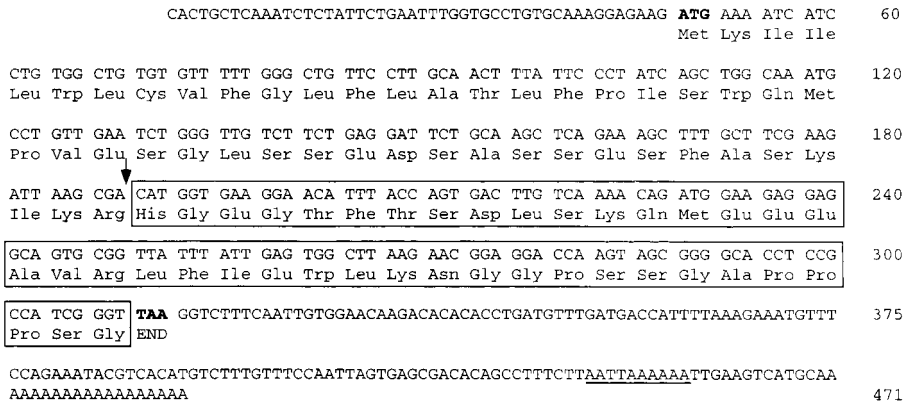

FIG. 3. **Exendin-4 cDNA and predicted amino acid sequence.** Shown is the nucleotide sequence of the entire cloned exendin-4 cDNA and the deduced amino acid sequence of the exendin-4 precursor protein comprising the mature exendin-4 peptide and a 45-amino acid N-terminal extension peptide. The sequence of the mature exendin-4 peptide peptide, as demonstrated previously by peptide sequencing, is *boxed*. The dibasic cleavage site of the precursor protein is indicated by an *arrow*, and the putative mRNA polyadenylation signal is *underlined*.

annealing temperature, 59 °C) using the sense primer 5′-TCGGAACCGCTCTCCAAAAGG-3′ derived from the sequence of the PHI/VIP-intervening peptide and the antisense RACE primer 2 resulted in a product of approximately 360 bp encoding the full Gila monster VIP sequence and approximately 280 bp of 3′ untranslated sequence. The sequence of the Gila monster VIP was confirmed using fewer PCR cycles by virtue of the nondegenerate sense primer from the PHI encoding cDNA sequence and the antisense primer from the 3′-untranslated region. All PCR products were subcloned into the pCR2.1 vector (TA cloning kit, Invitrogen, Carlsbad, CA) and sequenced with vector specific primers using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and an automated 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Southern Blot Analysis—Gila monster genomic DNA was isolated from the liver using a genomic DNA purification Kit (Qiagen, Santa Clarita, CA). The DNA was digested with restriction enzyme *Bam*HI, *Eco*RI, or *Hin*dIII, size fractionated on a 0.7% agarose gel (10 mg/lane), and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was probed with ^{32}P random prime-labeled cDNA probes specific for the coding regions of helodermin (265 bp), exendin-4 (263 bp), Gila monster PACAP (122 bp), VIP (247 bp), and GLP-1 (255 bp). The hybridization conditions and buffer were the same as those described for Northern blot analysis. Following hybridization the membranes were washed twice for 15 min under high stringency conditions (0.5× SSC, 0.1% SDS at 50 °C). The blots were analyzed using a phoshorimager, the image analysis software MacBAS, and the Fujix Pictography 3000 digital image printer (Fuji Photo Film U. S. A., Inc.). The size of the hybridizing DNA fragments was estimated using a 1-kb DNA ladder (Life Technologies, Inc.).

Cross-species Northern Blots and Screening of Mammalian Libraries—Using the above-described 265-bp helodermin and 263-bp exendin-4-specific ^{32}P random prime-labeled cDNA probes, cross-species Northern blots and mammalian libraries were screened for potential mammalian homologues to these peptides. Northern blot analysis was performed as described above, loading 2.5 μg of poly(A)$^+$ RNA per lane for various rat tissues, including brain, pancreas, intestine, and lung. A human genomic library (Lambda FIX II vector, Stratagene) and a rat brain cDNA library (Lambda ZAP II vector, Stratagene) (approximately 750×10^3 plaques/library) were screened according to the manufacturer's instructions. Nitrocellulose membranes were hybridized and washed for both Northern blots and libraries under low stringency conditions (hybridization for 14 h at 37 °C; 10× Denhardt's solution, 2× SSC, and 200 mg/ml denatured salmon sperm DNA; and washing twice at room temperature for 15 min with 2× SSC, 0.1% SDS).

RESULTS

This study was undertaken to identify potential mammalian homologues for the reptilian peptides helodermin and exendin-4 suspected from previous immunohistochemical studies and biological actions of these peptides at the VIP/PACAP and GLP-1 receptors and to determine the evolutionary relationship between these reptilian peptides and the highly homologous mammalian peptides, VIP/PACAP and GLP-1. To this end, we cloned the cDNAs encoding helodermin and exendin-4 from the Gila monster salivary gland and determined the tissue distribution of the two reptilian peptides within the Gila monster. Furthermore, we identified the reptilian homologues to mammalian PACAP, VIP, and GLP-1 and attempted to find mammalian homologues to helodermin and exendin-4.

To examine whether the reptilian peptides helodermin and exendin-4 are evolutionarily closely related to mammalian PACAP/VIP and GLP-1, respectively, we cloned their cDNAs from a Gila monster salivary gland cDNA library. The screening of approximately 750×10^3 plaques with oligonucleotide probes derived from the primary amino acid sequences of helodermin and exendin-4 resulted in more than 100 positive clones for both helodermin and exendin-4. Plaque purification and sequencing of at least 15 positive clones for both helodermin and exendin revealed that almost all clones contained the respective full-length cDNA. The structure of the helodermin cDNA is depicted in Fig. 2 and represents the consensus sequence of three independent clones. The helodermin cDNA is 440 bp in length and contains a single open reading frame of 252 bp, 27 bp of 5′ untranslated sequence, and 161 bp of 3′ untranslated sequence. The cDNA encodes the full amino acid

```
Helodermin      MKSILWLCVF GLLIATLFPV SWQMAIKSRL SSEDSETDQR L-FESKRHSD
Exendin-4       MKILWLCVF GLFLATLFPI SWQMPVESGL SSEDSASSES FASKIKRHGE

Helodermin      AIFTEEYSKL LAKLALQKYL ASIL--GSRT SPPPPSR   84
Exendin-4       GTFTSDLSKQ MEEEAVRLFI EWLKNGGPSS GAPPPSG   87
```

FIG. 4. **Alignment of the predicted amino acid sequences of the precursor peptides for helodermin and exendin-4.** The predicted amino acid sequence deduced from the entire coding sequence of helodermin and exendin-4 cloned cDNAs are shown using the single letter amino acid symbols and aligned for maximal homology. Conserved identical amino acid residues are *boxed*, showing the significant homology between the two N-terminal extension peptides (*boldface*). The start of the sequences of the mature peptides is indicated by an *arrow*.

sequence of the helodermin peptide as it was predicted by peptide sequencing (13) plus three additional C-terminal amino acids (Pro-Ser-Arg) before the stop codon. Furthermore, the cDNA encodes a 44-amino acid extension peptide of unknown function. The cloning of the exendin-4 cDNA revealed that it is very similar to the helodermin cDNA in size as well as in structure. The exendin-4 cDNA is 470 bp long and contains a single open reading frame of 261 bp, 48 bp of 5′ untranslated sequence, and 143 bp of 3′ untranslated sequence before the poly(A)$^+$ tail (Fig. 3). The exendin-4 cDNA that we cloned is nearly identical to the one previously published (1) and encodes the full exendin-4 peptide sequence as well as a 45-amino acid N-terminal extension peptide. Surprisingly, an alignment of the coding sequences of the helodermin and exendin-4 cDNAs revealed a high homology between the two N-terminal extension peptides in addition to their similarity in length. For the first 32 N-terminal amino acids, the homology between the two extension peptides is 84% (Fig. 4). Northern blot analysis of RNA from the Gila monster salivary gland revealed a single hybridizing transcript of approximately 500 bp for both the helodermin and the exendin-4 cDNA probe (Fig. 5), confirming that the cDNAs we isolated were close to full-length.

To determine whether helodermin and exendin-4 are expressed in tissues outside the salivary gland within the Gila monster, the highly sensitive method of RT-PCR was performed on a variety of Gila monster tissues. Using helodermin and exendin-4 cDNA-specific primers and 35 rounds of PCR, the PCR products of the expected size (265 bp for helodermin and 263 bp for exendin-4) were detected only in the salivary gland. RT-PCR analysis of all other tissues were negative (Fig. 6). The 28S:18S ribosomal RNA ratio was at least 1:1 for all tissues (data not shown).

To identify the reptilian homologues to mammalian PACAP, VIP, and GLP-1, partial cDNA sequences encoding the full or partial amino acid sequences of Gila monster PACAP, PHI, VIP, glucagon, and GLP-1 were cloned by PCR using the degenerate primers and PCR conditions described under "Experimental Procedures." Using degenerate primers derived from the N-terminal and C-terminal ends of the PACAP peptide, a 122-bp PCR product was amplified from the Gila monster brain encoding 26 amino acids of the middle portion of the Gila monster PACAP peptide. This peptide fragment was 96–100% homologous to the corresponding fragments of the PACAP peptides from the other indicated species (Fig. 7). A 182-bp PCR product representing a partial cDNA sequence encoding the 23 C-terminal amino acids of the Gila monster PHI, a 20-amino acid PHI/VIP-intervening peptide (not shown), and the first two C-terminal amino acids of Gila monster VIP was amplified from Gila monster lung using degenerate primers derived from the conserved N-terminal amino acids of PHI and VIP. The 23 C-terminal amino acids of the Gila monster PHI are 56% homologous to human PHI and are followed by an alanine instead of the glycine that would be expected if this peptide, like mammalian PHI, was amidated (Fig. 7). The C-terminal portion of the Gila monster VIP was cloned using a 3′-RACE strategy and nested PCR with primers derived from the sequence of the Gila

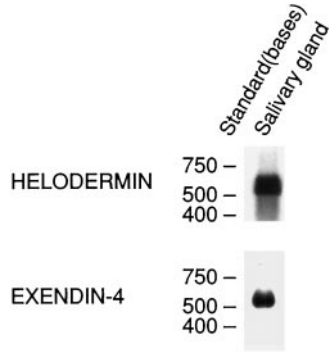

FIG. 5. **Northern blot analysis of Gila monster salivary gland.** Northern blot hybridization of 0.05 μg of mRNA from Gila monster salivary gland with a 265-bp-long helodermin-specific (*top panel*) or a 263-bp-long exendin-4-specific (*bottom panel*) ^{32}P random prime-labeled cDNA probe. RNA size markers are shown on the *left*. For both helodermin and exendin-4, a single hybridizing transcript of approximately 500 bases was detected.

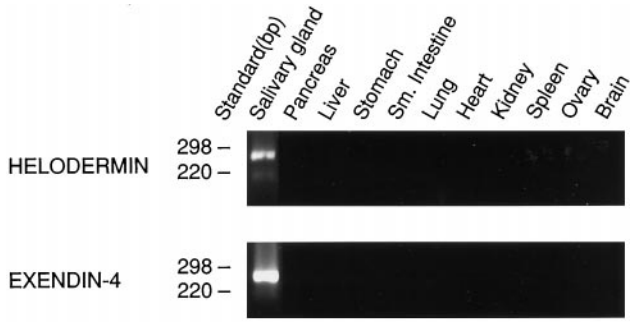

FIG. 6. **RT-PCR of Gila monster tissues.** Poly(A)$^+$ RNA (0.05 μg) from Gila monster salivary gland and total RNA (5 μg) from all other tissues were reverse transcribed and amplified during 30 cycles of PCR using helodermin (*top panel*) and exendin-4 (*bottom panel*) cDNA-specific primers. The PCR products of the expected size (265 and 263 bp, respectively) could only be detected in the salivary gland. DNA size markers are shown on the *left*.

monster PHI and the PHI/VIP intervening peptide (see "Experimental Procedures"). Ultimately, a 360-bp PCR product encoding the full Gila monster VIP peptide and approximately 280 bp of 3′ untranslated sequence was amplified from Gila monster lung. The Gila monster VIP is 67–75% homologous to the VIP peptides from the other indicated species (Fig. 7). The structure of the Gila monster VIP is somewhat unique in that the amino acids in the positions 4, 5, 9, 24, and 25 are unique to Gila monster VIP and do not occur in the VIP peptides from other species. The last amino acid of the Gila monster VIP is a glycine, which could theoretically allow for the α-amidation of the peptide. This glycine was followed by a dibasic cleavage site. Using degenerate primers derived from the N-terminal end of glucagon and the C-terminal end of GLP-1, we further amplified a 255-bp PCR product encoding the 17 C-terminal amino acids of the Gila monster glucagon, a 28-amino acid glucagon/GLP-1 intervening peptide, and the 23 N-terminal

PACAP

```
lizard:    -------DSYSRYRKQMAVKKYLAAVLGKRYKQ-----
human:     HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK*
rat:       HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK*
catfish:   HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK*
```

PHI

```
lizard:    ----IFNKAYRKVLGQLSARKYLHSLM
human:     HADGVFTSDFSKLLGQLSAKKYLESLM*
rat:       HADGVFTSDYSRLLGQISAKKYLESLI*
chicken:   HADGIFTSVYSHLLAKLAVKRYLHSLI
```

VIP

```
lizard:    HSDGIFTDSYSRYRKQMAVKKYLAAVLG
human:     HSDAVFTDNYTRLRKQMAVKKYLNSILN*
rat:       HSDAVFTDNYTRLRKQMAVKKYLNSILN*
chicken:   HSDAVFTDNYSRFRKQMAVKKYLNSVLT*
alligator: HSDAVFTDNYSRFRKQMAVKKYLNSVLT*
dogfish:   HSDAVFTDNYSRIRKQMAVKKYINSLLA*
```

GLUCAGON

```
lizard:    ------------YLDTRRAQDFVQWLMNT
human:     HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
rat:       HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
chicken:   HSQGTFTSDYSKYLDSRRAQDFVQWLMST
trout:     HSEGTFSNDYSKYQEERMAQDFVQWLMNS
```

GLP-1

```
lizard:    HAEYERHADGTYTSDISSYLEGQ-------------
human:     HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGR*
rat:       HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGR*
chicken:   HSEFERHAEGTYTSDITSYLEGQAAKEFIAWLVNGR*
trout:     ------HADGTYTSDVSTYLQDQAAKDFVSWLKSGR
```

FIG. 7. **Comparison of primary amino acid sequences of Gila monster PACAP, PHI, VIP, glucagon, and GLP-1 with their homologues from different species.** Partial or full peptide amino acid sequences of Gila monster PACAP, PHI, VIP, glucagon, and GLP-1 were deduced from partial cDNA sequences that were cloned using degenerate oligonucleotide primers derived from the consensus sequence of the peptides known in other species. The Gila monster peptide amino acid sequences are aligned with their homologues from other species using single letter amino acid symbols. Conserved identical amino acid residues are shown in *boldface*, and C-terminal α-amidation of the peptide, when known, is indicated by a *star*.

amino acids of the Gila monster GLP-1. The Gila monster glucagon peptide fragment was 88 and 94% homologous to chicken and human glucagon, respectively (Fig. 7). The N-terminal part of Gila monster GLP-1 was 83% homologous to chicken GLP-1. The sequence of the Gila monster glucagon, glucagon/GLP-1 intervening peptide (not shown), and GLP-1 was consistent with the sequences previously published (1), except for one amino acid in the Gila monster GLP-1. The amino acid at position 11 in the Gila monster GLP-1 was found to be a threonine instead of an arginine.

To confirm the likelihood that the messages for helodermin, PACAP, and VIP, on the one hand, and exendin-4 and GLP-1, on the other, are the product of separate genes, Southern blot hybridization of Gila monster genomic DNA with cDNA probes specific for the coding regions of Gila monster helodermin, PACAP, VIP, exendin-4, and GLP-1 was performed. These

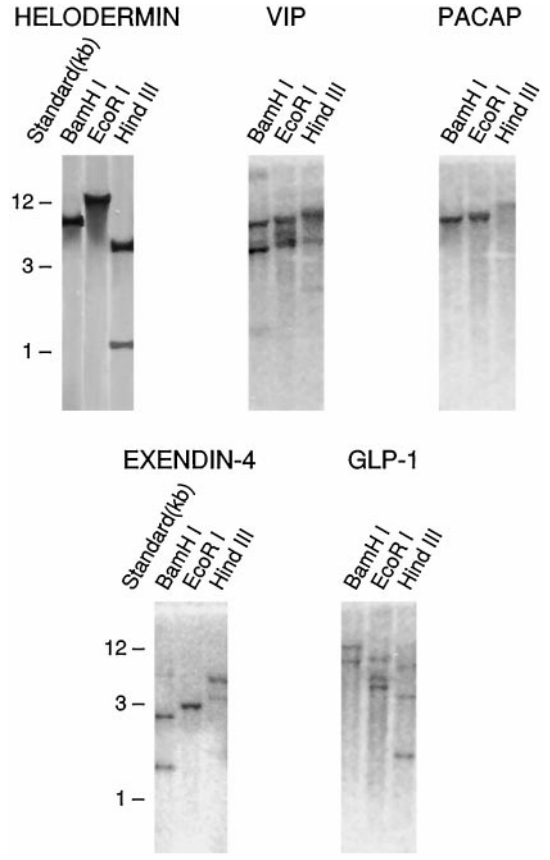

FIG. 8. **Southern blot analysis of Gila monster genomic DNA.** Southern blot hybridization of Gila monster genomic DNA (10 mg/lane) digested with the indicated restriction enzymes and hybridized with the indicated ^{32}P random prime-labeled cDNA probes: Gila monster helodermin, VIP, or PACAP (*top*) and Gila monster exendin-4 or GLP-1 (*bottom*). DNA size markers are indicated on the *left*.

blots of Gila monster genomic DNA cut separately with three rare restriction enzymes revealed that each of these probes hybridized in a unique pattern, suggesting that the message for each of these peptides arises from a separate gene (Fig. 8).

In an extensive search for mammalian homologues to reptilian helodermin and exendin-4, a 265-bp helodermin-specific and a 263-bp exendin-4-specific cDNA probe were used to perform cross-species hybridization studies. Although the message for these secreted peptides should be abundant, as demonstrated earlier by the Gila monster salivary gland Northern blot and cDNA library screen, cross-species Northern blots using 5 μg poly(A)$^+$ RNA from different rat and human tissues (including salivary gland, pancreas, small intestine, and brain) probed with high specific activity ($>1 \times 10^9$ cpm/μg) ^{32}P random prime-labeled probes under both extremely low and high stringency conditions failed to identify hybridizing transcripts. In the event that these probes, derived from a lower evolved reptile, were insufficiently homologous to the sought-after mammalian equivalent for Northern blotting, increased sensitivity was sought by screening several cDNA libraries constructed from rat and human tissues suspected to express either helodermin or exendin-4 on the basis of their presence in the reptile salivary gland, expected physiologic interaction with the receptors for VIP/PACAP and GLP-1 in brain and pancreas, or previously demonstrated immunoreactivity in brain. Screening ($>750 \times 10^3$ plaques/library) under low hybridization conditions also failed to identify hybridizing plaques. Screening of a rat brain cDNA library using the same helodermin- and exendin-4-derived degenerate oligonucleotide probes that successfully identified helodermin and exendin-4

cDNA clones from the lizard salivary gland was also unsuccessful. To exclude the unlikely possibility that the cDNA libraries screened were not sufficiently representative, genomic libraries from rat and human were screened (>750 × 10^3 plaques/library) with the same high specific activity helodermin and exendin-4 probes under low stringency conditions without success.

DISCUSSION

The present study was undertaken to determine whether there are evolutionarily conserved mammalian homologues to the reptilian peptides helodermin and exendin-4 that are suspected to exist on the basis of the reptilian peptides affinity and functional interaction with the mammalian VIP_2 (15) and GLP-1 receptors (22–24), respectively, as well as previous immunohistochemical localization studies suggesting their presence in several mammalian tissues (18–21). Alternatively, in the event that helodermin and exendin-4 could not be found in mammals, the possibility that they could be the reptilian precursors to VIP/PACAP and GLP-1, as suggested by others (3, 14) was also examined. The cloning of the nearly full-length helodermin and exendin-4 cDNAs revealed that these two cDNAs are very similar in length and structural organization to one another but not to the cDNAs of the mammalian peptides PACAP, VIP, or GLP-1. Expression of the message for helodermin and exendin-4 peptides was restricted to the Gila monster salivary gland. The expression of the messages for the peptides PACAP, VIP, and GLP-1 in the Gila monster was demonstrated by the cloning of a partial cDNA sequence for each of the respective peptides from the appropriate tissue outside the salivary gland. Southern blot analysis revealed that helodermin, exendin-4, PACAP, VIP, and GLP-1 exist on different genes in the Gila monster. Attempts to demonstrate the presence of mammalian homologues for helodermin and exendin-4 by low stringency hybridization methods using probes derived from either helodermin or exendin-4 cloned cDNA sequences were unsuccessful.

The considerable homology in amino acid sequence between helodermin and mammalian PACAP and VIP, on the one hand, and exendin-4 and GLP-1, on the other hand, has led to the classification of the two reptilian peptides into the VIP/glucagon peptide family; the ability of helodermin to stimulate biologic actions through the mammalian VIP_2 receptor and the ability of exendin-4 to stimulate biologic actions through the mammalian GLP-1 receptor have led to the hypothesis that helodermin and exendin-4 might be the evolutionary precursors to mammalian PACAP/VIP or GLP-1 (3, 14). To test this hypothesis, we cloned the cDNAs of helodermin and exendin-4. The helodermin cDNA was 440 bp long and simply structured, encoding the helodermin peptide and a 44-amino acid extension peptide. Using high pressure liquid chromatography purification of helodermin from the Gila monster venom and subsequent peptide sequencing (13), the primary structure of helodermin was described as comprising 35 amino acids with C-terminal amidation. Although all 35 amino acids that were predicted by peptide sequencing are encoded by the helodermin cDNA, the cDNA predicts three additional amino acids (Pro-Ser-Arg) before the stop codon and therefore a 38-amino acid peptide. This is not necessarily a contradiction, because peptide processing enzymes that can cleave the peptide after a proline, the proline endopeptidases, have been described (36, 37). However, the cDNA does not encode a glycine that could serve as substrate for peptide α-amidation and therefore does not support the finding that helodermin is amidated at the C terminus. Another possible explanation for this discrepancy would be a degradation of the helodermin peptide in the Gila monster venom that was used for the purification of helodermin. Of note, the peptide helospectin I (a peptide that is 85% homologous to helodermin, has very similar biologic actions, and is believed to have evolved from the same ancestral lizard venom peptide as helodermin (3)) has been described by peptide sequencing as a 38-amino acid, non-amidated peptide (38). The marked difference in size and structural organization between the helodermin cDNA and the PACAP and VIP cDNAs (26, 27, 29, 30) argues that the reptilian peptide helodermin is not, despite the homology in primary amino acid sequence and similar biologic actions, the precursor of mammalian PACAP or VIP. The exendin-4 cDNA was found to be 470 bp in length, encoding the full exendin-4 peptide and a 45-amino acid N-terminal extension peptide. The cDNA sequence is fully consistent with the sequence predicted by peptide sequencing, including C-terminal amidation, as well as the sequence of the exendin-4 cDNA cloned independently (1). Although size and structure of the exendin-4 cDNA is markedly different from the glucagon/GLP-1 cDNA in both higher evolved and more primitive species (33–35), again arguing that exendin-4 is not the precursor to GLP-1, it is strikingly similar to the helodermin cDNA. The cDNAs are similar in size and encode, in addition to the helodermin and exendin-4 peptide, respectively, an N-terminal extension peptide of almost exactly the same size (44 *versus* 45 amino acids). Alignment of the two N-terminal extension peptides show a high degree homology between them. The similarity between the helodermin and exendin-4 cDNAs in size and structure suggests their evolution from a common ancestor gene by gene duplication. The conservation of the sequences of the N-terminal extension peptides may indicate that they have an as yet unknown biologic function.

Because the structure of the cDNAs suggested that helodermin and exendin-4 are not the precursors of mammalian PACAP/VIP and GLP-1, respectively, but represent a distinct family of peptides, we sought to investigate the expression of helodermin and exendin-4 in different tissues within the Gila monster to characterize the physiologic role of these peptides in the Gila monster. Screening a large variety of different Gila monster tissues, we failed to detect the expression of either peptide in any other tissue outside the salivary gland. This is consistent with the inability of others to detect exendin-4 in Gila monster intestine, liver, and pancreas by Northern blot analysis using an exendin-4 cDNA probe (1). Therefore, the peptides helodermin and exendin-4 seem to have a highly specialized function as components of the venom. The cloning of peptide fragments from Gila monster brain, lung, and pancreas that are highly homologous to mammalian PACAP, VIP, and GLP-1, respectively, identified the true precursors of mammalian PACAP, VIP, and GLP-1 in the Gila monster, the likely physiologic agonists at the Gila monster VIP and GLP-1 receptors. This was not an unexpected result, because all three peptides have been shown to occur in even lower species, such as fish (28, 32, 35). The sequences of the Gila monster glucagon, glucagon/GLP-1 intervening peptide (not shown), and GLP-1 were consistent with the sequences previously published (1), except for one amino acid in the Gila monster GLP-1. The amino acid in position 11 in the Gila monster GLP-1 was found to be a threonine instead of an arginine. A threonine in this position seems to be more likely, because this amino acid is conserved in other species, both higher evolved and more primitive (Fig. 5). The demonstration by Southern blot analysis that helodermin is encoded on a different gene than the Gila monster PACAP and VIP peptides, and is therefore subject to different gene regulation, is in concert with the different tissue expression patterns of these peptides in the Gila monster. The same is true for exendin-4 and Gila monster GLP-1, as shown previously (1).

The discovery of bioactive nonmammalian peptides, especially in frog skin, has led in some cases to the discovery of novel mammalian peptides with similar biologic activities (6, 7, 39). Our search for as yet unknown mammalian homologues to the reptilian peptides helodermin and exendin-4 was unsuccessful. This result is consistent with the apparently highly specialized function of both helodermin and exendin-4 within the Gila monster as components of the venom. Notably, bombesin and ranatensin display a tissue distribution in the frog that is similar to that of their mammalian counterparts GRP and neuromedin B in mammals (8–10). It is, however, inconsistent with the detection of helodermin-like immunoreactivity in a variety of mammalian tissues (18–21). One weakness of the immunohistochemical studies is a possible cross-reaction of the antibodies used. In fact, cross-reaction of anti-helodermin antibodies used in these studies with PHI, calcitonin-like substances, and androgen-binding protein have been reported (40–42).

It seems most likely that the high affinity and biological activity of helodermin and exendin-4 at the mammalian VIP_2 and GLP-1 receptors, respectively, are the result of convergent evolution. Other examples of convergent evolution of venom peptides from nonmammalian species toward mammalian receptors have been described previously (5, 11). A vasodilator peptide named maxadilan was isolated from salivary gland lysates of the sand fly. Although maxadilan shares only a few identical amino acids with the mammalian PACAP peptide, it binds with high affinity to the mammalian PACAP type 1 receptor and is able to stimulate cAMP production through this receptor with nearly the same efficacy and potency as PACAP (5). The relationship of helodermin to PACAP/VIP and exendin-4 to GLP-1 seem to be most similar to the relationship between safarotoxin S6C, a component of a snake venom, and mammalian endothelin I. Although the two peptides are 72% homologous in amino acid sequence and share very similar pharmacological properties at the mammalian endothelin receptor (43–45), the difference in size and structure of the two cDNAs argues against the two peptides being evolutionarily related and for convergent evolution (11).

In summary, we have shown that the two reptilian peptides helodermin and exendin-4 are evolutionarily close to each other but distinct from mammalian PACAP/VIP and GLP-1, respectively, with which they share biologic actions. The physiologic role of helodermin and exendin-4 within the Gila monster seems to be confined to being a component of the venom, and the physiologic agonists of the Gila monster VIP and GLP-1 receptors seem to be the Gila monster PACAP/VIP and GLP-1 peptides. These results, together with the unsuccessful search for mammalian homologues, argue that the biological activities of helodermin and exendin-4 on the mammalian receptors are the result of convergent evolution and that the existence of as yet unknown mammalian homologues seems unlikely, although it cannot be excluded.

Acknowledgments—We thank Dr. Benjamin Gonzales of the Adobe Mountain Wildlife Center and Jeffrey Howland from the Non-Game Branch of the Arizona Game and Fish Department (Phoenix, AZ) for their assistance in providing the Gila monster tissues necessary to conduct this study.

REFERENCES

1. Chen, Y. E., and Drucker, D. J. (1997) *J. Biol. Chem.* **272,** 4108–4115
2. Bertaccini, G. (1976) *Pharmacol. Rev.* **28,** 127–177
3. Raufman, J.-P. (1996) *Reg. Pept.* **61,** 1–18
4. Schweitz, H., Vigne, P., Moinier, D., Frelin, C., and Lazdunski, M. (1992) *J. Biol. Chem.* **267,** 13928–13932
5. Moro, O., and Lerner, E. A. (1997) *J. Biol. Chem.* **272,** 966–970
6. McDonald, T. J., Jörnvall, H., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S. R., and Mutt, V. (1979) *Biochem. Biophys. Res. Commun.* **90,** 227–233
7. Minamino, N., Kangawa, K., and Matsuo, H. (1983) *Biochem. Biophys. Res. Commun.* **114,** 541–548
8. Spindel, E. R., Gibson, B. W., Reeve, J. R., and Kelly, M. (1990) *Proc. Natl. Acad. Sci.* **87,** 9813–9817
9. Krane, I. M., Naylor, S. L., Helin-Davis, D., Chin, W. W., and Spindel, E. R. (1988) *J. Biol. Chem.* **263,** 13317–13323
10. Nagalla, S. R., Gibson, B. W., Tang, D., Reeve, J. R., Jr., and Spindel, E. R. (1992) *J. Biol. Chem.* **267,** 6916–6922
11. Takasaki, C., Itoh, Y., Onda, H., and Fujino, M. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1527–1533
12. Raufman, J.-P., Jensen, R. T., Sutliff, V. E., Pisano, J. J., and Gardner, J. D. (1982) *Am. J. Physiol.* **242,** G470–G474
13. Hoshino, M., Yanaihara, C., Hong, Y.-M., Kishida, S., Katsumaru, Y., Vandermeers, A., Vandermeers-Piret, M.-C., Robberecht, P., Christophe, J., and Yanaihara, N. (1984) *FEBS Lett.* **178,** 233–239
14. Eng, J., Kleinman, W. A., Singh, L., Singh, G., and Raufman, J.-P. (1992) *J. Biol. Chem.* **267,** 7402–7405
15. Lutz, E. M., Sheward, W. J., West, K. M., Morrow, J. A., Fink, G., and Harmar, A. J. (1993) *FEBS Lett.* **334,** 3–8
16. Gourlet, P., De Neef, P., Woussen-Colle, M.-C., Vandermeers, A., Vandermeers-Piret, M.-C., Robberecht, P., and Christophe, J. (1991) *Biochim. Biophys. Acta* **1066,** 245–251
17. Shima, K., Sakakibara, H., and Said, S. I. (1996) *Regul. Pept.* **63,** 99–103
18. Robberecht, P., De Graef, J., Woussen, M.-C., Vandermeers-Piret, M.-C., Vandermeers, A., De Neef, P., Cauvin, A., Yanaihara, C., Yanaihara, N., and Christophe, J. (1985) *Biochem. Biophys. Res. Commun.* **130,** 333–342
19. Robberecht, P., De Neef, P., Vandermeers, A., Vandermeers-Piret, M.-C., Svoboda, M., Meuris, S., De Graef, J., Woussen-Colle, M.-C., Yanaihara, C., Yanaihara, N., and Christophe, J. (1985) *FEBS Lett.* **190,** 142–146
20. Grunditz, T., Persson, P., Hakanson, R., Absood, A., Böttcher, G., Rerup, C., and Sundler, F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1357–1361
21. Bjartell, A., Persson, P., Absood, A., Sundler, F., and Hakanson, R. (1989) *Regul. Pept.* **26,** 27–34
22. Fehmann, H. C., Jiang, J., Schweinfurth, J., Wheeler, M. B., Boyd, A. E., and Göke, B. (1994) *Peptides* **15,** 453–456
23. Göke, R., Fehmann, H.-C., Linn, T., Schmidt, H., Krause, M., Eng, J., and Göke, B. (1993) *J. Biol. Chem.* **268,** 19650–19655
24. Schepp, W., Schmidtler, J., Riedel, T., Dehne, K., Schusdziarra, V., Holst, J. J., Eng, J., Raufman, J.-P., and Classen, M. (1994) *Eur. J. Pharmacol.* **269,** 183–191
25. Han, J. H., Stratowa, C., and Rutter, W. J. (1987) *Biochemistry* **26,** 1617–1625
26. Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R. R., Stibbs, H. H., Arimura, A., and Fujino, M. (1990) *Biochem. Biophys. Res. Commun.* **166,** 81–89
27. Ogi, K., Kimura, C., Onda, H., Arimura, A., and Fujino, M. (1990) *Biochem. Biophys. Res. Commun.* **173,** 1271–1279
28. McRory, J. E., Parker, D. B., Ngamvongchon, S., Sherwood, N. M. (1995) *Mol. Cell. Endocrinol.* **108,** 169–177
29. Nishizawa, M., Hayakawa, Y., Yanaihara, N., and Okamoto, H. (1985) *FEBS Lett.* **183,** 55–59
30. McFarlin, D. R., Lehn, D. A., Moran, S. M., MacDonald, M. J., and Epstein, M. L. (1995) *Gene* **154,** 211–213
31. Wang, Y., and Conlon, J. M. (1993) *Peptides* **14,** 573–579
32. Dimaline, R., Young, J., Thwaites, D. T., Lee, C. M., and Thorndyke, M. C. (1988) *Ann. N. Y. Acad. Sci.* **527,** 621–623
33. Drucker, D. J., and Asa, S. (1988) *J. Biol. Chem.* **263,** 13475–13478
34. Heinrich, G., Gros, P., Lund, P. K., Bentley, R. C., and Habener, J. F. (1984) *Endocrinology* **115,** 2176–2181
35. Irwin, D. M., and Wong, J. (1995) *Mol. Endocrinol.* **9,** 267–277
36. Taylor, W. L., and Dixon, J. E. (1980) *Biochem. Biophys. Res. Commun.* **94,** 9–15
37. Andrews, P. C., Hines, C. M., and Dixon, J. E. (1980) *Biochemistry* **19,** 5494–5500
38. Parker, D. S., Raufman, J.-P., O'Donohue, T. L., Bledsoe, M., Yoshida, H., and Pisano, J. J. (1984) *J. Biol. Chem.* **259,** 11751–11755
39. Feurle, G. E., Hamscher, G., Kusiek, R., Meyer, H. E., and Metzger, J. W. (1992) *J. Biol. Chem.* **267,** 22305–22309
40. Vandermeers, A., Gourlet, P., Vandermeers-Piret, M.-C., Cauvin, A., De Neef, P., Rathe, J., Svoboda, M., Robberecht, P., and Christophe, J. (1987) *Eur. J. Biochem.* **164,** 321–327
41. Tsutsumi, Y., Kamoshida, S., Iguchi, K., Mochizuki, T., and Yanaihara, N. (1990) *Regul. Pept.* **31,** 11–21
42. Karn, R. C., and Russell, R. (1993) *Biochem. Genet.* **31,** 307–319
43. Williams, D. L., Jones, K. L., Pettibone, D. J., Lis, E. V., and Clineschmidt, B. V. (1991) *Biochem. Biophys. Res. Commun.* **175,** 556–561
44. Koham, D. E., Padilla, E., and Hughes, A. K. (1993) *Am. J. Physiol.* **265,** F670–F676
45. Iwasaki, S., Homma, T., Matsuda, Y., and Kon, V. (1995) *J. Biol. Chem.* **270,** 6997–7003