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Purification, Amino Acid Sequence, Synthesis, and Receptor Selectivity of Alligator Gastrin

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Gastrin-like immunoreactive peptides were extracted from the gastric antrum of the American alligator (*Alligator mississippiensis*) and purified by fractionation using C_{18} Sep-Paks, Sephadex G-50, pH stable C_8 reversed-phase HPLC, and C_{18} reversed-phase HPLC. Three major immunoreactive peaks were purified and found to correspond to 49, 45, and 34 residue peptides by microsequence analysis. The amino acid sequence of the largest peptide was DWLASLSQDQ KHLISKFLPH IYGELAN QEN YWQEDDALHD HDYPGWMDF-amide. The two smaller peptides corresponded to carboxyl-terminal 45 and 34 residue fragments of the 49 residue peptide. The putative proteolysis of the 49 residue peptide to the two shorter peptides occurs at cleavage sites that are unusual in biosynthetic processing. Mass spectral analysis confirmed the molecular weights that were predicted from the amino acid sequences, thus revealing the absence of any post-translational modifications, such as sulfation. Although the three alligator gastrins resemble mammalian cholecystokinin in having a tyrosine residue in the seventh position from the carboxyl terminus, this tyrosine is apparently nonsulfated as in turtle gastrin. When tested by radioreceptor assay, a synthetic replicate of alligator gastrin-49 exhibited a gastrin-like pattern of biological activity on mammalian CCK-A and CCK-B receptors. Comparison of the amino acid sequences of known peptides revealed that alligator gastrin is most similar to turtle gastrin (76% identical), followed by frog gastrin (51% identical), chicken gastrin (49% identical), and human gastrin (12% identical). These similarities closely reflect vertebrate phylogeny and support the hypothesis that functionally distinct gastrins evolved from CCK in early tetrapods. However, gastrin evolved via different mechanisms in the mammalian lineage (mechanism unknown) versus the amphibian and reptilian/avian lineages, in which two different single nucleotide base changes can account for the separate evolution of amphibian gastrin and reptilian/avian gastrin.

Cholecystokinin (CCK) is a gastrointestinal hormone and a neurotransmitter in the central and peripheral nervous systems in mammals. All biologically active forms of CCK have the same carboxyl-terminal tetrapeptide amide sequence (–Trp–Met–Asp–Phe–NH_2), which is the molecular center of biological activity of the full peptide. This tetrapeptide amide is also present in the stomach hormone gastrin, explaining the observation that CCK and gastrin share qualitative activities at the same target cells and only differ quantitatively in their potencies. The functionally important difference between the two distinct mammalian peptides is determined by the position of the tyrosine residue adjacent to the active tetrapeptide

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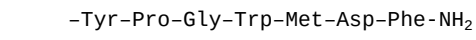
domain. In all known forms of CCK, there is a tyrosine in the seventh position from the carboxyl terminus, whereas in mammalian gastrins, the tyrosine is in the sixth position from the carboxyl terminus. In addition, this tyrosine residue is always sulfated in CCK but may or may not be sulfated in mammalian gastrins. The sharing of the identical molecular center of biological activity between CCK and gastrin suggests that these peptides form a molecular family and may have evolved from a common ancestor (Dockray, 1977; Vigna, 1986).

Considerable evidence has been provided in support of the hypothesis that a CCK-like peptide is the ancestral member of the CCK/gastrin molecular family (reviewed in Dockray, 1977; Vigna, 1986; Johnsen and Rehfeld, 1993; Dimaline and Dockray, 1994; Rourke *et al.*, 1997). A peptide with sulfated tyrosine residues at both the sixth and seventh positions from the carboxyl terminus has been identified in extracts of the neural ganglion of the urochordate *Ciona intestinalis* and named cionin (Johnsen and Rehfeld, 1990). Cionin thus resembles both mammalian CCK and the sulfated form of mammalian gastrin; when tested in mammalian systems, cionin exhibited a CCK-like pattern of biological activity (Schjoldager *et al.*, 1991). It has been shown immunochemically that the brain and gut of the agnathan lampreys *Lampetra fluviatilis* and *L. tridentata* contain CCK-like peptides (Holmquist *et al.*, 1979). The brain of the spiny dogfish *Squalus acanthias* has been demonstrated recently to contain CCK-8 identical in amino acid sequence to that of most mammals, as well as identical to chicken, turtle, and frog CCK (Johnsen, 1995). Also, two distinct CCKs, described as being different in amino acid sequence from all other known CCKs, have been reported in the rainbow trout *Oncorhynchus mykiss* (Jensen and Johnsen, 1994). The first evidence for duplication of the ancestral CCK-encoding gene comes in amphibians from the demonstration that bullfrog (*Rana catesbeiana*) intestine and stomach express two different peptides of the CCK family (Johnsen and Rehfeld, 1992; Johnsen, 1994); both the intestinal and the gastric peptides have a sulfated tyrosine residue seven positions from the carboxyl terminus and thus are equipotent when tested on mammalian CCK-A receptors (Nielsen *et al.*, 1996). In addition, the substitution in the sixth position from the carboxyl terminus of an alanine residue in bullfrog gastrin for the methionine in bullfrog CCK results in an eightfold greater potency of bullfrog gastrin for stimulating bullfrog gastric acid secretion relative to bullfrog CCK (Nielsen *et al.*, 1996). This demonstrates a degree of specificity of bullfrog gastrin for a gastrin target organ, although the difference in potency between bullfrog gastrin and CCK for the bullfrog receptors is not as great as the difference in potency between mammalian gastrin and CCK for mammalian CCK-A and -B receptors. In reptiles, the turtle (*Pseudomys scripta*) expresses intestinal CCK and a novel putative gastrin-like peptide in the stomach, which contains a nonsulfated tyrosine residue in the seventh position from the carboxyl terminus (Johnsen and Rehfeld, 1992; Johnsen, 1994). It is notable that turtle gastrin also has a proline residue in the sixth position from the carboxyl terminus. In birds, the chicken peptides resemble the turtle peptides with the exception that chicken gastrin is expressed in both sulfated and nonsulfated forms (Dimaline *et al.*, 1986). Although the sulfate moiety in sulfated chicken gastrin is present on a tyrosine residue seven positions from the carboxyl terminus, it has been hypothesized that the proline residue in position six from the carboxyl terminus may produce a steric effect that lowers the activity of the peptide at the subtype of CCK receptor specific for CCK, the CCK-A receptor (Dimaline and Lee, 1990). The amino acid sequences of the CCKs and gastrins reported to date are shown in Table 1.

The amino acid sequence and bioassay information now available suggests that peptides exhibiting the biological properties of gastrin, i.e., ability to stimulate CCK-B/gastrin receptors but with low potency at the CCK-specific CCK-A receptor subtype, evolved multiple times by distinct mechanisms from an ancestral CCK-like peptide in the tetrapod lineage in vertebrate phylogeny. It appears that three distinct structural changes in CCK have resulted in the evolution of peptides with gastrin-like biological activities. First, in amphibia, a single nucleotide base change encoding the amino acid in the sixth position from the carboxyl terminus of CCK resulted in a peptide with gastrin-like bioactivity. Second, in a common ancestor of reptiles and birds, nonsulfation of an otherwise CCK-like peptide and substitution of proline (which can occur by a single nucleotide base change in the codon for alanine) in the sixth position from the carboxyl

TABLE 1
Carboxyl-Terminal Heptapeptide Amino Acid Sequences of Chordate Cholecystokinins and Gastrins

Peptide	Animal
	Mammalian gastrin
	Human, pig, chicken, turtle, shark, frog cholecystokinin
	Chicken gastrin
	Turtle gastrin
	Bullfrog gastrin
	Caerulein (frog skin)
	*Ciona* cholecystokinin (cionin)

Note. The positions of the amino acid residues are numbered unconventionally from the carboxyl terminus. S, SO_3H; *, Tyr exists in both sulfated and unsulfated forms.

terminus appeared (in birds, the seventh position tyrosine exists in both sulfated and nonsulfated forms; the presence of proline in the sixth position appears to reduce greatly the potency of sulfated chicken gastrin at CCK-A receptors) (Dimaline and Lee, 1990). Third, in mammals, gastrin evolved by a mechanism distinct from those seen in amphibia and in reptiles / birds. The critical tyrosine residue was shifted from the seventh to the sixth position from the carboxyl terminus; thus, both sulfated and nonsulfated mammalian gastrins have very low potency at CCK-A receptors. Substitution of alanine (amphibia) and proline (reptiles and birds) in the sixth position from the carboxyl terminus and shifting of the tyrosine residue from the seventh to the sixth position (mammals) are modifications requiring nucleotide base changes in the DNA encoding the peptides, whereas nonsulfation is a loss of a post-translational modification mechanism.

The purpose of the present study was to test the hypothesis that expression of a nonsulfated gastrin typified by seventh position tyrosine and sixth position proline is a derived character shared by extant reptiles and birds. We determined the amino acid sequences of three forms of gastrin in the American alligator *Alligator mississippiensis* (a representative of the reptilian order Crocodylia), synthesized alligator gastrin-49, and determined the affinity of alligator gastrin-49 for CCK-A and CCK-B receptors. Portions of the present results have been published previously in abstract form (Vigna and Reeve, 1994).

METHODS

Animals. Alligator stomachs were obtained from 1-year-old animals (body length 4 feet) of both sexes raised at a commercial alligator farm in Louisiana and fed a commercial diet *ad libitum*. The animals were killed by a blow to the head, stomachs were removed immediately, and the gastric antrum was separated, frozen on dry ice, and stored at −80° until extraction.

Extraction and purification. One liter of 0.1% ammonium bicarbonate was brought to a boil and small pieces of frozen alligator antrum were added slowly so not to cool the extraction medium. A total of 82.6 g of antrum was extracted. The extract was boiled for 10 min, cooled to room temperature, and then placed on ice. The ice-cold extract was then centrifuged at 4° for 45 min at 10,000g_{max}. The supernatant (900 ml) was

then pumped through two sets of three C_{18} SEP-PAK cartridges (Waters, Milford, MA) in series that were previously washed with 20 ml of acetonitrile followed by 30 ml of 0.1% ammonium bicarbonate. The extract was pumped through the SEP-PAKs at a rate of 1.2 ml/min using syringe pumps. When the C_{18} packing within the third SEP-PAK in each series was saturated with adsorbed extract as determined by the green color of the extract, pumping of the extract was halted and the three SEP-PAK cartridges in series were replaced with new cartridges. A total of 12 SEP-PAK cartridges were needed to adsorb the entire extract. After loading the extract on the SEP-PAK cartridges, the cartridges were washed with 20 ml of 0.1% ammonium bicarbonate and then eluted with 70% acetonitrile in 0.1% ammonium bicarbonate yielding 14.5 ml. This concentrated extract was then diluted to 100 ml with 0.1% ammonium bicarbonate and loaded onto a column containing 21 of Sephadex G-50 Superfine (Pharmacia, Piscataway, NJ) previously equilibrated with and eluted with 0.1% ammonium bicarbonate. Gastrin-like immunoreactivity (gastrin-like-ir) in eluate fractions was determined by radioimmunoassay as described below and the fractions containing the greatest amount of gastrin-like-ir were pooled (pool volume = 38.4 ml) and loaded onto a semipreparative (1×25 cm), pH stable, reversed-phase Vydac C_8 HPLC column (The Separations Group, Hesperia, CA) previously equilibrated with 0.1% ammonium bicarbonate, pH 8.2. The column was eluted by a linear gradient of 0–50% acetonitrile in 0.1% ammonium bicarbonate in 100 min. Eluate fractions representing each of three peaks of gastrin-like-ir were separately pooled, diluted threefold in 0.1% trifluoroacetic acid (TFA), and separately fractionated on an analytical (4.6×250 mm) Vydac C_4 reversed-phase HPLC column (The Separations Group) previously equilibrated in 0.1% TFA, pH 2. The columns were eluted by a linear gradient of 0–50% acetonitrile in 0.1% TFA in 100 min. Single peaks of gastrin-like-ir eluted from each column were characterized by amino acid, microsequence, and mass spectral analysis.

Cholecystokinin/gastrin radioimmunoassay. Antiserum 5135 was a kind gift from Drs. John H. Walsh and Grace Rosenquist. The radioimmunoassay based on this antiserum is specific for the carboxyl terminus of cholecystokinin and gastrin (Rosenquist and Walsh, 1980). Radioimmunoassay in tissue extracts and column fractions was performed as previously described (Rosenquist and Walsh, 1980) using ^{125}I-synthetic human (Leu^{15})-gastrin-17-I as tracer and synthetic porcine CCK8 as standard.

Amino acid analysis, sequence determination, and mass spectrometry. For amino acid analysis, purified peptides (approximately 100 pmol) were dried in hydrolysis tubes and then hydrolyzed in 6 *N* HCl at 110° for 18 hr under vacuum in a nitrogen atmosphere. The hydrolysates were derivitized with phenylisothiocyanate (PITC). The PITC amino acids were then analyzed on a reversed-phase HPLC column using a sodium acetate-acetonitrile gradient.

Peptide microsequence determination was performed at the UCLA Protein Microsequence Facility. Samples of purified peptides were subjected to Edman degradation on a Porton 2090E Sequencer. The phenylthiohydantoin amino acid derivatives were analyzed by HPLC.

For mass spectral analysis, fast atom bombardment (FAB) spectra were obtained [VG ZAB-SE, Fisons Instruments, VG Analytical, Manchester, UK, 11/250 data system, 8 kV accelerating potential, zenon bombarding gas at 8 kV and 1 mA, and a mass resolution of 600 (10% valley, M/DM)] from 1- to 2-μl aliquots of dried HPLC fractions redissolved in 0.1% TFA in water that were applied to the static FAB probe tip onto which had already been placed 1–2 μl of liquid matrix [*meta*-nitrobenzylalcohol/thioglycerol/TFA (100/100/1)]. Spectra were obtained by scanning from m/z 6200 to 3000 and about 10 scans were collected from each sample into a multichannel analyzer. The data were smoothed, centroided, and mass measured using cesium iodide ion clusters for calibration. The accuracy of the mass measurements carried out in this manner is typically better than ± 1 Da. Electrospray ionization (ESI) mass spectra were obtained [Perkin–Elmer Sciex, Thornhill, Canada, API III; 4.5 kV ion spray voltage, spray nebulization with hydrocarbon-depleted air ("zero" grade air, 40 psi, 0.6 liter/min; Zero Air Generator, Peak Scientific, Chicago, IL), nitrogen curtain gas (0.6 liter/min) from the vapors of liquid gas, and mass resolution so the isotopes of the polypropylene glycol (PPG)/NH_4^+ singly charged ion at m/z 906 were resolved with 40% valley] from 10-μl aliquots of dried HPLC fractions redissolved in water/acetonitrile/formic acid (50/50/0.1) that were injected into an infusion line connected to the ion source. The same

solvent mixture was flowing through the infusion line (10 µl/min) and data were obtained (orifice voltages of 50 and 90 yielded essentially identical results) by scanning from m/z 500 to 2000. The resulting spectra were summed then background subtracted and mass measured using for calibration eight PPG solution signals (the singly charged ions at m/z 58.99, 326.25, 906.67, 1254.92, 1545.13, 1863.34, and 2010.47, and the doubly charged ion at m/z 520.4) obtained from a separate introduction of calibrant [a mixture of PPG 425, 1000, and 2000 (3.3×10^{-5}, 1×10^{-4}, and 2×10^{-4} *M*, respectively) in water/methanol (1/1, v/v) containing 2 m*M* ammonium formate and 0.1% acetonitrile]. Deconvolution of the series of multiply charged sample ions and calculation of peptide molecular weights were achieved with the Hypermass computer program supplied with the instrument. Under the data acquisition parameters employed the accuracy of mass assignment of singly charged ions was $<\pm 0.05$ Da, which translates into an expected accuracy of $<\pm 0.5$ Da for peptides in the 4- to 6-kDa molecular size range.

Peptide synthesis. Alligator gastrin-49 was synthesized using FMOC strategies on an Advanced ChemTech Multiple Peptide Synthesizer Model 396, cleaved at room temperature (cleavage mixture: 90% trifluoroacetate, 5% thioanisol, 3% ethanedithiol, 2% anisole), purified by reversed-phase HPLC using a linear gradient of acetonitrile for elution, and characterized by mass spectral analysis as described above and by high performance capillary electrophoresis (Beckman Model 2200).

Radioreceptor assay. Radioreceptor assays using mammalian CCK-B receptors (guinea pig cerebral cortical membranes) and CCK-A receptors (rat pancreatic membranes) were performed by modifications of the methods described by Innis and Snyder (1980). The CCK analog dTyr–Gly–Asp–Tyr(SO_3H)-Nle–Gly–Trp–Nle–Asp–Phe-amide (Research Plus, Bayonne, NJ) was iodinated for use as the receptor radioligand by incubating 5 µg of the peptide, 500 µCi of ^{125}I, and 2.5 µg of chloramine T in 30 µl of 200 m*M* phosphate buffer, pH 7.5 for 20 sec at room temperature. The reaction was stopped by addition of 50 µl of 50% glacial acetic acid and then the reaction products were purified by reversed-phase HPLC. The specific activity of the monoiodinated CCK analog peptide (^{125}I-labeled CCK-10 analog) was 1954 µCi/nmol. Guinea pigs and rats were killed and guinea pig cerebral cortices and rat pancreases were removed, extraneous tissue was trimmed away, and 10 vol (w/v) of 50 m*M* Tris–HCl, pH 7.4, containing 0.02% soybean trypsin inhibitor was added. The organs were minced with scissors and then homogenized using 5–10 passes of a motor-driven Teflon–glass tissue grinder. The pancreas homogenate was filtered through two layers of gauze and then the brain and pancreas homogenates were centrifuged for 20 min at 37,000g_{max} at 4°. The pellets were resuspended in the same Tris buffer and recentrifuged as before. These pellets were dispersed in incubation buffer using five passes of the tissue grinder. The incubation buffer consisted of 20 m*M* Hepes, 118 m*M* NaCl, 4.7 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.02% soybean trypsin inhibitor, 0.5% BSA, and 0.1% bacitracin, pH 6.5. The protein concentration of the membranes (before adding incubation buffer) was determined by the method of Bradford (1976). Freshly prepared membranes (75 µg protein) were incubated in plastic tubes in 1 ml of incubation buffer with 100 p*M* ^{125}I-labeled CCK-10 analog for 1 hr at 22°. Nonsaturable binding was determined in the presence of 1 µ*M* nonradioactive CCK-10 analog. All determinations were performed in triplicate. After incubation, triplicate 300-µl aliquots were removed from each tube and centrifuged at 10,000*g* in a microcentrifuge for 30 sec. The supernatants were aspirated to waste and 300 µl of ice-cold incubation buffer was added to the pellets. The tubes were then recentrifuged as before for 10 sec. The supernatants were aspirated to waste, the tips of the tubes containing the pellets were cut off, and the radioactivity in the tips was determined in a gamma scintillation counter.

Data analysis. Saturation binding curves were analyzed at equilibrium to determine the affinities of the tested peptides. Saturation binding values were analyzed by nonlinear regression using the program Prism (GraphPad Software, San Diego, CA) to obtain estimates of the concentrations of peptides resulting in one-half maximal inhibition of saturable binding (IC_{50}).

RESULTS

After fractionating the crude extract on SEP-PAK C_{18} cartridges followed by gel permeation chromatogra-

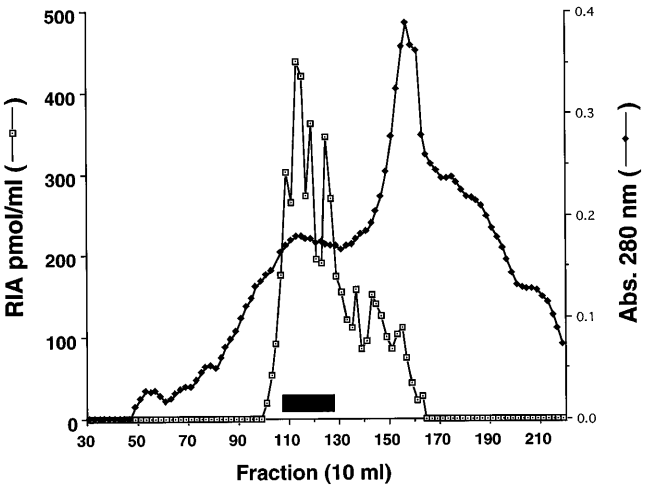


FIG. 1. Fractionation of alligator antral stomach extract by gel permeation chromatography on Sephadex G-50 Superfine. Ultraviolet absorbance at 280 nm (Abs 280 nm) and gastrin-like-ir (RIA pmol/ml) are plotted versus fraction number. The fractions containing gastrin-like-ir pooled for further analysis are shown by the solid bar.

phy on Sephadex G-50 Superfine, one broad peak of gastrin-like-ir was observed (Fig. 1). When the peak fractions (No. 107–129) of gastrin-like-ir from the Sephadex G-50 Superfine column were pooled and subjected to reversed-phase, pH stable C_8 HPLC fractionation at pH 8.2, three separate peaks of gastrin-like-ir were observed (Fig. 2, peaks A–C). When the individual peaks of gastrin-like-ir eluted from the pH stable C_8 column were separately fractionated using an analytical C_4 reversed-phase HPLC column at pH 2, single discrete peaks of gastrin-like-ir corresponding to major A_{220} peaks were obtained (Fig. 3). Attempts to purify additional molecular forms of alligator gastrin from fractions 130–160 (Fig. 1) were unsuccessful. The gastrin-like-ir eluting from this region of the column may be explained by peak nonsymmetry or by smaller molecular forms of alligator gastrin.

The amino acid analyses of the purified alligator gastrin-like-ir peaks A–C (Fig. 3) are shown in Table 2. Peak A corresponded to a peptide of 34 amino acid residues, peak B corresponded to a peptide of 45 amino acid residues, and peak C corresponded to a peptide of 49 amino acid residues. Amino acid sequence analysis of each peptide resulted in the sequences shown in Fig. 4. Each peptide washed out of the sequencer before the sequence analysis reached the carboxyl terminus, presumably because of the highly hydrophobic nature of the carboxyl-terminal residues. Therefore, the carboxyl-terminal sequence of –Asp–Phe-NH_2 was assigned by comparison of the sequence obtained with the amino acid composition (Table 2), by mass spectral analysis (Table 3), and by the immunoreactivity of the peptides with antiserum No. 5135, which requires carboxyl-terminal Phe-NH_2 for immunoreactivity (Rosenquist and Walsh, 1980). The two smaller peptides corresponded to carboxyl-terminal fragments of the larger peptide. Mass spectral analysis

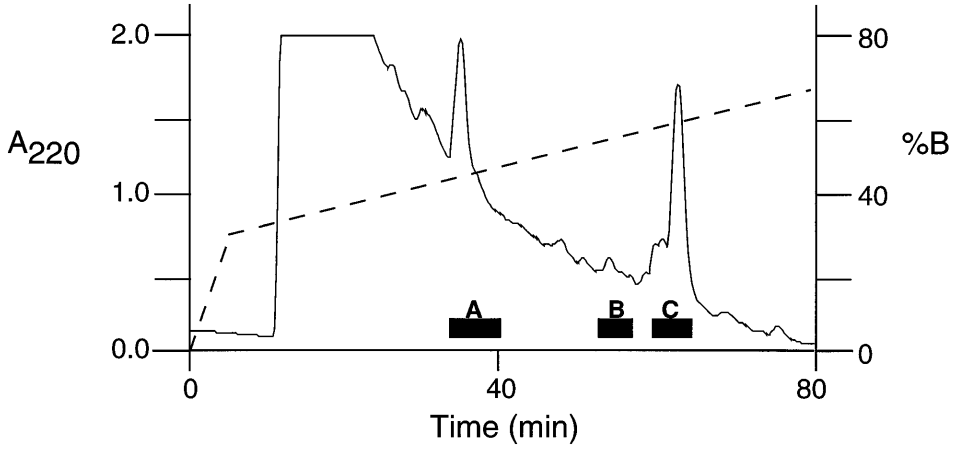


FIG. 2. HPLC fractionation of the pooled fractions (No. 107–129) from Fig. 1 on a semipreparative pH stable reversed-phase C_8 column. Ultraviolet absorbance at 220 nm (A_{220}) and the 50% acetonitrile gradient used to elute the column (%B) are plotted versus time. The fractions containing gastrin-like-ir pooled for further analysis are shown by the solid bars.

of each peptide showed a close correspondence between the mass calculated from the deduced amino acid sequences and the observed mass, thus confirming the sequence analysis and also demonstrating that the peptides were not post-translationally modified (Table 3). Searches in both the FAB and the electrospray data for signals from peptides exhibiting masses 80 Da larger than those found, corresponding to covalent attachment of a sulfate group to a hydroxyl function, were negative.

The largest form of alligator gastrin isolated, gastrin-49, was synthesized so that sufficient material was available to examine the biological properties of the peptide. When tested using guinea pig cerebral cortical membranes predominantly expressing the CCK-B receptor subtype, synthetic alligator gastrin-49 concentration-dependently inhibited the saturable binding of ^{125}I-labeled CCK-10 analog in parallel with human gastrin-17-I and CCK-10 analog (Fig. 5). The affinity of alligator gastrin-49 (IC_{50} = 46.8 n*M*) for the mammalian CCK-B receptor was approximately one-fourth that of the affinity of human gastrin-17-I (IC_{50} = 12.9 n*M*). Synthetic alligator gastrin-49 did not significantly inhibit the saturable binding of ^{125}I-labeled CCK-10 analog to mammalian CCK-A receptors in rat pancreatic membranes at concentrations up to 1 µ*M* (Fig. 5).

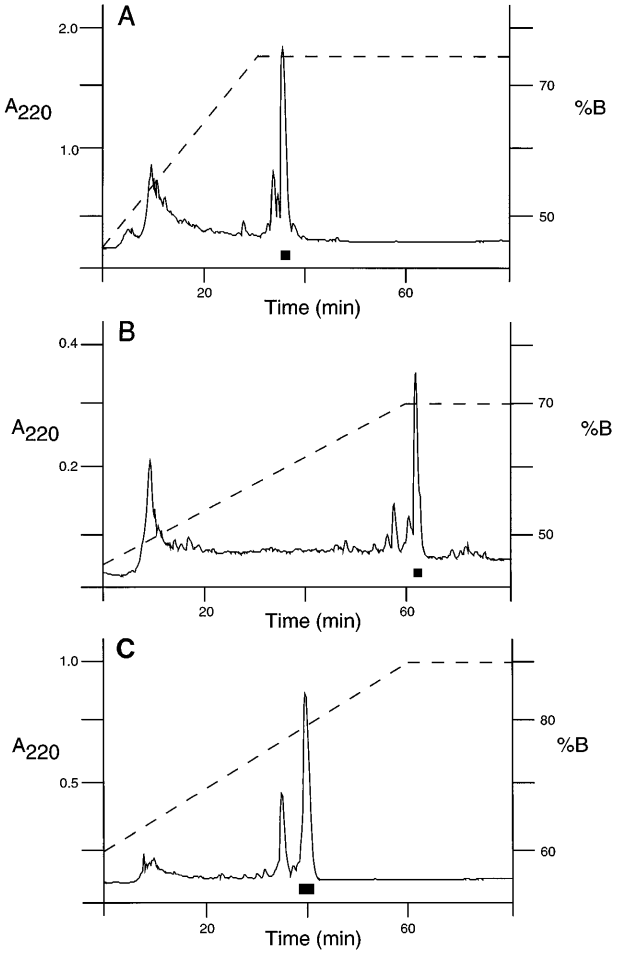


FIG. 3. HPLC fractionation of separately pooled fractions containing peaks A–C from Fig. 2 on an analytical reversed-phase C_4 column. Ultraviolet absorbance at 220 nm (A_{220}) and the 50% acetonitrile gradient used to elute the column (%B) are plotted versus time. The fractions containing gastrin-like-ir used for microsequence analysis, amino acid analysis, and mass spectral analysis are shown by the solid bars.

TABLE 2
Amino Acid Analysis Results for Purified Peaks A–C of Alligator Gastrin

Amino acid	Peak A observed (expected)	Peak B observed (expected)	Peak C observed (expected)
Cys	0.0 (0)	0.0 (0)	0.0 (0)
Asp	6.5 (7)	8.4 (8)	9.6 (9)
Glu	4.7 (5)	7.0 (7)	7.0 (8)
Ser	0.3 (0)	3.1 (3)	3.0 (3)
Gly	2.4 (2)	2.3 (2)	2.1 (2)
His	2.3 (3)	3.4 (4)	3.6 (4)
Arg	0.4 (0)	0.0 (0)	0.2 (0)
Thr	0.3 (0)	0.0 (0)	0.3 (0)
Ala	2.0 (2)	2.2 (2)	2.8 (2)
Pro	2.0 (2)	2.4 (2)	2.4 (2)
Tyr	1.9 (3)	2.7 (3)	2.8 (3)
Val	0.5 (0)	0.3 (0)	0.1 (0)
Met	0.6 (1)	0.5 (1)	0.5 (1)
Ile	0.9 (1)	1.8 (2)	1.6 (2)
Leu	2.6 (3)	4.4 (5)	5.1 (7)
Phe	1.8 (2)	2.0 (2)	1.9 (2)
Lys	0.8 (1)	1.9 (2)	2.1 (2)
Trp*	n.d. (2)	n.d. (2)	n.d. (2)
Total amino acids	34	45	49
Amount (nmol/ml)	6.1	2.7	10.0

Note. n.d., not determined due to loss during hydrolysis.

DISCUSSION

The current extraction and purification of gastrin-like-ir from the alligator gastric antrum confirms a previous report of the immunocytochemical localiza-

KFLPHIYGELANQENYWQEDDALHDHDYPGWMDF-amide Peak A

SLSQDQKHLISKFLPHIYGELANQENYWQEDDALHDHDYPGWMDF-amide Peak B

DWLASLSQDQKHLISKFLPHIYGELANQENYWQEDDALHDHDYPGWMDF-amide Peak C

FIG. 4. Amino acid sequence analysis of alligator gastrin-like-ir peaks A–C from Fig. 3. The sequences of the amino acids underlined were obtained by direct automated microsequence analysis. The sequences of the remainder of the amino acids shown were obtained as described in the text.

tion of gastrin-like-ir in the alligator gastric antrum (Buchan *et al.*, 1983). The amino acid sequences of the alligator gastrins are also consistent with the observation that gastrin cells in the alligator gastric antrum only reacted with antisera specific for the carboxyl terminus of mammalian gastrin and not with N-terminal specific antisera (Buchan *et al.*, 1983) because the only region of significant amino acid sequence similarity between alligator gastrin and mammalian gastrins is at the carboxyl terminus (Fig. 6).

The 45 and 34 residue forms of alligator gastrin could be derived by proteolysis of the larger 49 residue form. The peptide bonds (A_{46}–S_{45} and S_{35}–K_{34}) in alligator gastrin-49 that are cleaved to produce the smaller forms are unusual because most prohormones are processed by cleavage at di- and monobasic sites (Bjφrnskov *et al.*, 1992). Bullfrog and turtle gastrins have also been isolated in several forms with the smaller peptides corresponding to carboxyl-terminal fragments of the larger form in which the putative cleavage sites are also unusual in that they are not di- or monobasic residues (Johnsen and Rehfeld, 1992).

TABLE 3

Comparison of the Observed Masses, Determined by Mass Spectroscopy, of Purified Alligator Gastrins versus Their Predicted Masses Calculated from Their Amino Acid Sequences

Peptide	Observed mass (Da) FAB (MH^+)	Observed mass (Da) Electrospray (M)	Calculated mass (Da)
aGastrin-34	4195.3[a]	4194.4 ± 0.3[b]	4194.5
aGastrin-45	5433.8[c]	5431.7 ± 0.6[b]	5431.9
aGastrin-49	5916.2[d]	5917.5 ± 0.6[b]	5917.4

[a] Estimated measurement error: +0.35 to −0.5.

[b] Values represent the average of data obtained from three separate sample introductions.

[c] Estimated measurement error: +0.8 to −0.9.

[d] Estimated measurement error: +1.0 to −1.2.

However, the sites of cleavage in bullfrog and turtle gastrins do not correspond to those in alligator gastrin and are not the same pairs of amino acids found at the cleavage sites in alligator gastrin. Also, four forms of chicken gastrin appear to be processed by different

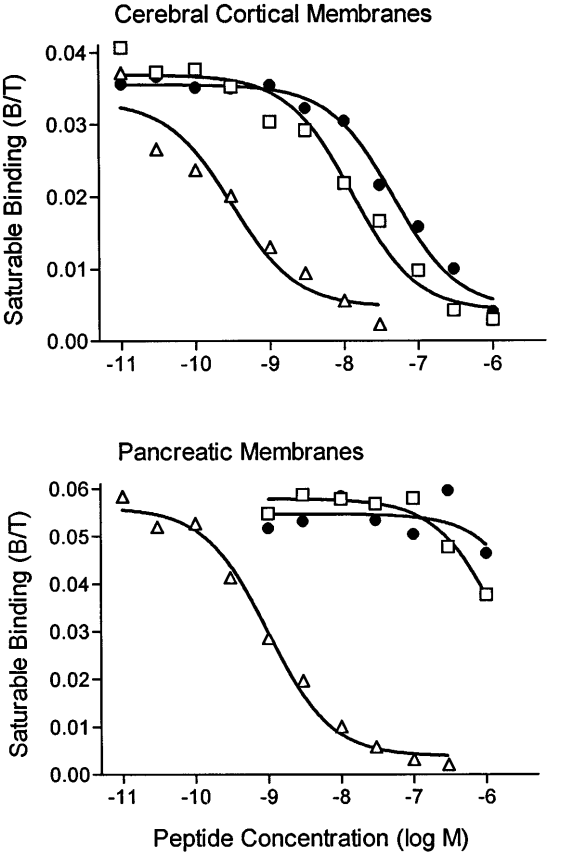


FIG. 5. Radioreceptor assay of synthetic alligator gastrin-49 on mammalian CCK-A receptors (rat pancreatic membranes) and mammalian CCK-B receptors (guinea pig cerebral cortical membranes). The inhibition of the saturable binding of ^{125}I-labeled CCK-10 analog by nonradioactive CCK-10 analog (△), human gastrin-17-I (□), and synthetic alligator gastrin-49 (●) is plotted versus the concentration of the peptides.

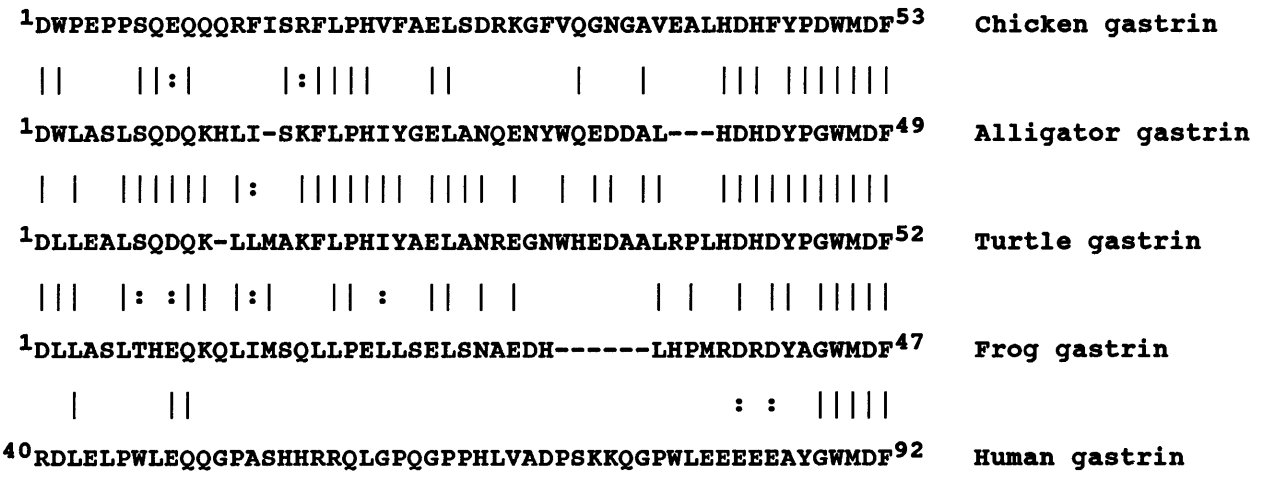


FIG. 6. Comparisons of the amino acid sequences of vertebrate gastrins. Identities (|) and the functionally conservative substitutions D / E, R / K, T / S, and L / I (:) are indicated. Sources of sequences: chicken gastrin (Dimaline *et al.,* 1986; Bjφrnskov *et al.,* 1992); turtle and frog gastrin (Johnsen and Rehfeld, 1992); human gastrin (Boel *et al.,* 1983).

mechanisms than the bullfrog and reptilian gastrins because cleavage of the chicken gastrins occurs at post-phenylalanine bonds (Bjφrnskov *et al.,* 1992). If these patterns of cleavage represent the normal biosynthesis of these peptides rather than extraction artifacts, it appears that post-translational processing in this peptide family is highly species dependent (Johnsen, 1994).

The amino acid sequence identities among the known vertebrate gastrins, including alligator gastrin, closely reflect phylogenetic relationships (Fig. 6). Alligator gastrin is most similar to turtle gastrin (76% identical), is about equally similar to frog gastrin (51% identical) and chicken gastrin (49% identical), and is least similar to human gastrin (12% identical, primarily at the carboxyl-terminal pentapeptide active site domain of the peptides).

The greater affinity of alligator gastrin-49 for mammalian CCK-B receptors than for mammalian CCK-A receptors is consistent with the finding that the alligator peptide is nonsulfated. We have also recently demonstrated that alligator gastrin exhibits a very low affinity for a third vertebrate CCK receptor subtype, the CCK-X receptor (Oliver and Vigna, 1997). Synthetic alligator gastrin-49 tested on homologous alligator receptors showed a higher affinity for the alligator stomach CCK-B-like receptor than for the gallbladder CCK-A receptor, confirming the specificities of alligator gastrin for mammalian CCK-A and -B receptor subtypes (Oliver and Vigna, 1997).

The distribution and evolution of vertebrate CCKs and gastrins are summarized in Fig. 7. We follow the phylogenetic hypothesis proposed by Gauthier *et al.* (1988) and Benton (1991) with regard to the relationships within the amniota: Mammalia, Chelonia (turtles), Lepidosauria (lizards, snakes, and tuatara), Crocodylia (crocodiles, alligators), and Aves (birds). The critical structural features determining CCK-like versus gastrin-like patterns of biological activity are noted by showing the position of the tyrosine residue from the carboxyl terminus (Y_7 vs Y_6), by showing whether the tyrosine residue is sulfated (Y_{7S}), and by showing the residue in the sixth position from the carboxyl terminus (Y_6, M_6, A_6, or P_6). It seems clear that CCK-like peptides are likely to be the primitive, ancestral members of this family (box 1 in Fig. 7; Rourke *et al.,* 1997). It appears that the first evolution of a separate gene for a peptide with a gastrin-like pattern of bioactivity occurred in the amphibian lineage after its divergence from the other tetrapods (box 2 in Fig. 7). Subsequently, two different mutations must have occurred leading to the evolution of two separate types of gastrin-like molecules, one in the mammalian lineage (box 3 in Fig. 7) and another in the reptilian / avian lineage (box 4 in Fig. 7). In mammals, the tyrosine residue is shifted to the sixth position from the carboxyl terminus, resulting in a large decrease in the affinity of the peptide at CCK-A receptors and therefore specificity for CCK-B / gastrin receptors (Vigna *et*

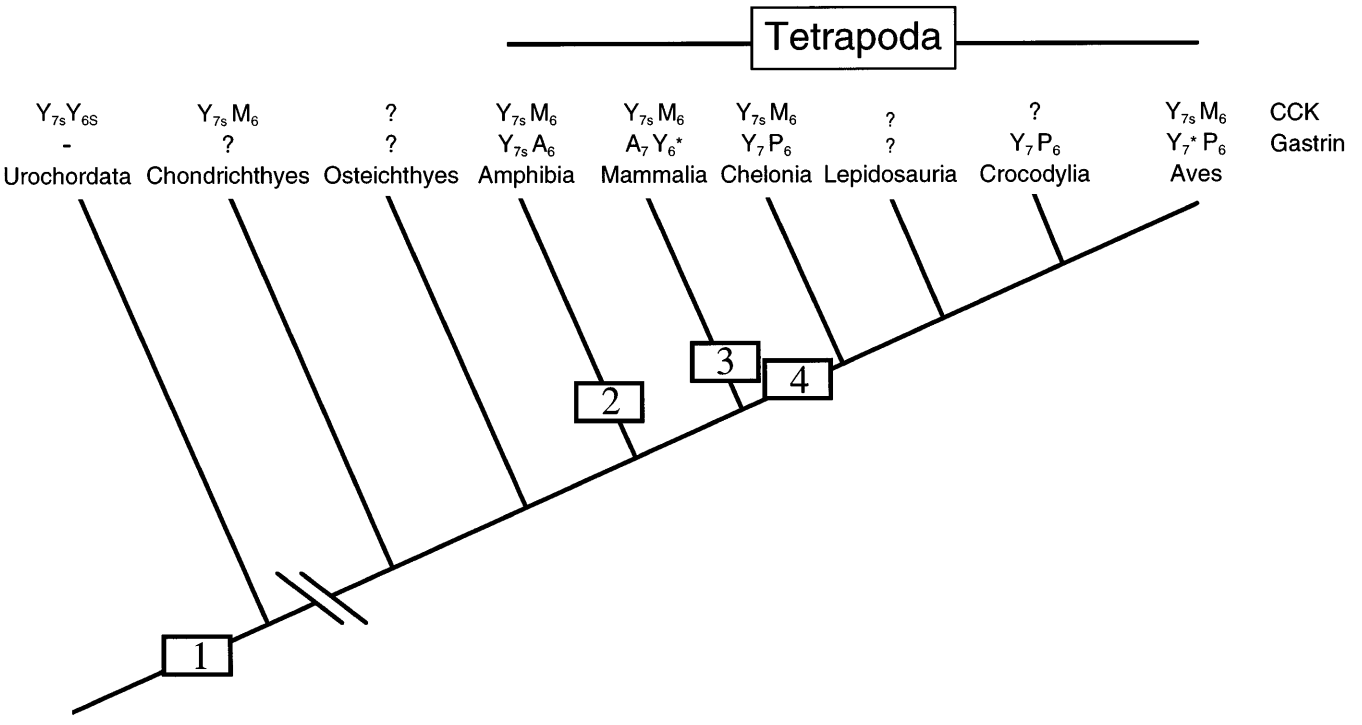


FIG. 7. Distribution of types of gastrin and cholecystokinin (CCK) in vertebrate phylogeny. Character states for gastrin and CCK are noted above each group. The critical structural character states determining CCK-like versus gastrin-like patterns of biological activity are noted by showing the position of the tyrosine residue from the carboxyl terminus (Y_7 vs Y_6), by showing whether the tyrosine residue is sulfated (Y_{7s}), and by showing the residue in the sixth position from the carboxyl terminus (Y_6, M_6, A_6, or P_6). The hypothesized origins of derived gastrin and CCK character states are noted as follows: (1) Y_{7s} CCK and gastrin are primitive, (2) Y_7A_6 gastrin evolved in the amphibian lineage, (3) A_7Y_6 evolved in the mammalian lineage, and (4) Y_7P_6 evolved in the reptilian / avian lineage. −, not present; ?, not reported; / /, agnathans are omitted because no CCK or gastrin amino acid sequences have been determined in an agnathan; ∗, mammalian and avian gastrins occur in both nonsulfated (shown) and sulfated forms.

al., 1986). This change apparently occurred early in the mammalian lineage because it is found in both eutherian mammals and in a marsupial, the Eastern Grey kangaroo (*Macropus giganteus giganteus*), a representative of the primitive prototherian mammals (Johnsen and Shulkes, 1993). In the lineage leading to modern reptiles and birds, on the other hand, it appears that a single nucleotide base change may have occurred in the codon for alanine in the sixth position from the carboxyl terminus, as found in frog gastrin, resulting in the substitution of proline in this position as seen in turtle, alligator, and chicken gastrins. The sixth position proline in turtle and alligator gastrins may not play a significant role in determining the biological activities of these peptides because the tyrosine residue in the seventh position in these peptides is nonsulfated, a feature which by itself leads to gastrin-like biological properties. However, chicken gastrin occurs in both sulfated and nonsulfated forms, and it is thought that the proline in the sixth position of sulfated chicken gastrin results in a steric effect that reduces the potency of the peptide at CCK-A receptors, and thus is a crucial feature in determining the biological properties of the peptide (Dimaline and Lee, 1990).

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