Elasmobranchs express separate cholecystokinin and gastrin genes

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ABSTRACT The peptide hormone gastrin was long believed to be specific for higher vertebrates, whereas its homologue, cholecystokinin (CCK), has been assumed to represent the original ancestor of the CCK/gastrin family. To trace the divergence of the CCK/gastrin family beyond birds, reptiles, and amphibians we have now examined sharks. Distinct CCK and gastrin peptides were identified in two shark species, the spiny dogfish (Squalus acanthias) and the porbeagle (Lamna cornubica). The corresponding genes and cDNAs were isolated and sequenced from the spiny dogfish. Comparison with several vertebrate species show that the CCK gene and peptide structures have been considerably more conserved than the corresponding gastrin structures. Alignment of the dogfish prepropeptides displays similarities that support the hypothesis that they share a common ancestor. Our findings move the CCK/gastrin family segregation back to at least 350 million years ago. This event must have occurred before, or perhaps during, the evolution of cartilaginous fishes, probably concomitant with the occurrence of gastric acid secretion.

Cholecystokinin (CCK) and gastrin are brain–gut peptides characterized by the common C terminus -Trp-Met-Asp-Phe-NH$_2$. This tetrapeptide amide constitutes the minimal structure necessary for biological activity of both hormones, although potencies are determined by the proximal N-terminal sequences. The two peptides were originally described in mammals, where they differ in the position of a Tyr residue: gastrin has Tyr in position 6 from the C terminus (1), whereas CCK has Tyr in position 7 (2) (Fig. 1). Expression of two distinct members of the CCK/gastrin family has also been described in the nonmammalian vertebrates chicken (3, 4) and bullfrog and slider turtle (5–7). These species all produce CCK with the C-terminal octapeptide identical to mammalian CCK and with pronounced overall similarity to the mammalian CCKs. The second type of peptide, isolated from the stomachs of the same species, also has Tyr in position 7 like CCK (3, 5, 8). However, even the C-terminal octapeptide region contains substitutions (Fig. 1), and the N-terminal extensions differ markedly from those of the CCKs. On the other hand, they display a high degree of mutual similarity. On the basis of structural features and their anatomical origin, the second peptides were named gastrins (3, 5). Subsequently, chicken gastrin (9) and bullfrog gastrin (10) were shown to have distinct bioactivity properties. Thus, in spite of the structural C-terminal resemblance, nonmammalian CCK and gastrin also constitute separate hormonal systems.

The common active site suggests that CCK and gastrin evolved from a common ancestor (11, 12). In search for the putative ancestor we isolated the octapeptide cionin from the protochordate Ciona intestinalis (13) (Fig. 1). Cionin appears as an obvious member of the CCK/gastrin family, which thus can be dated to very early in chordate evolution.

Several reports have described CCK/gastrin-like peptides in elasmobranchs (14–16). With the aim of further elucidating when separate CCK and gastrin peptides occurred during evolution, the present investigation was undertaken to characterize the CCK/gastrin peptides in two sharks, spiny dogfish (Squalus acanthias) and porbeagle (Lamna cornubica), as well as their genes in the spiny dogfish.

MATERIALS AND METHODS

Experimental Animals and Tissue Extractions. Spiny dogfish and porbeagle (or mackerel shark) were caught in the North Sea and kept iced on the fishing boat. Heads and guts were obtained shortly after landing. All tissues were frozen on solid CO$_2$, immediately after dissection and kept frozen until further processing. The frozen tissues were extracted in boiling water, followed by ice-cold 0.5 M acetic acid as previously described (5). For peptide purification 550 g of spiral intestine and 126 g of cerebrum from dogfish were subjected to extraction, while 230 g of spiral intestine and 30 g of brain (primary cerebrum) from porbeagle were employed. For the isolation of mRNA a single spiny dogfish was obtained live from the North Sea Museum (Hirtshals, Denmark).

Radioimmunoassays. The antisera 2609 and 2717 are both specific for the carboxyamidated C-terminal tetrapeptide common to CCK and gastrin (17, 18). Antiserum 2717 was used to

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Abbreviation: CCK, cholecystokinin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. for Squalus acanthias CCK: exon 1, Z97374; exon 2, Z97375; exon 3, Z97376; and cDNA, Z97359; for Squalus acanthias gastrin: exon 1, Z97371; exon 2, Z97372; exon 3, Z97373; and cDNA, Z97360).

FIG. 1. Selected members of the CCK/gastrin family. Note that mammalian gastrins have Tyr in position 6 from the C terminus, whereas CCK and nonmammalian gastrins all have Tyr in position 7. Thus even if cionin appears as a structural hybrid of CCK and mammalian gastrin octapeptides, it is unlikely to be the immediate predecessor of mammalian gastrins. CCK O-sulfation of the Tyr residue is shown by *; partial sulfation is indicated by (+).

Mammalian gastrin Cionin CCK-8 Bullfrog gastrin-8 Sliderturtle gastrin-8 Chicken gastrin

<table>
<thead>
<tr>
<th>Mammalian gastrin</th>
<th>- Ala - Tyr$^*$ - Gly - Trp - Met - Asp - Phe-NH$_2$</th>
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</tr>
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<td>Sliderturtle gastrin-8</td>
<td>- Asp - Tyr$^*$ - Pro - Gly - Trp - Met - Asp - Phe-NH$_2$</td>
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<tr>
<td>Chicken gastrin</td>
<td>- Tyr$^+$ - Pro - Asp - Trp - Met - Asp - Phe-NH$_2$</td>
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characterize the peptides present in dogfish intestine and monitor their purification, while antiserum 2609 was used for the remaining extracts. Depending on the tissue, the antiserum G160 (19) or 92128 (J.F.R., unpublished work), which both are specific for sulfated CCK, was used for further characterization.

**Analytical Peptide Chromatography.** Gel chromatography was performed using two different systems: a 1 × 100 cm column packed with Sephadex G-50 superfine (Pharmacia) as previously described (5), and a 10 × 300 mm Superdex Peptide column (Pharmacia) equilibrated at room temperature with 20 mM sodium phosphate (pH 8.0) containing 0.25 M NaCl at a flow rate of 0.25 ml/min. Fractions were collected at 1-min intervals. Calibration was performed by including 125I-labeled albumin (Y0) and 2-13CaNaCl (Y1). The elution constants (Kd) of peaks eluting at Yc are calculated as Kd = (Yc−Y0)/(Yc−Y0).

Anion exchange was performed on an FPLC system (Pharmacia) using a 5 × 50 mm MonoQ column (Pharmacia) as previously described (5).

**Peptide Purification.** Thawed extracts were centrifuged 30 min at 10,000 × g before they were pumped onto a 26 × 100 mm HiLoad Q Sepharose column (Pharmacia) equilibrated with (i) 50 mM Tris-HCl (pH 8.2) (porbeagle intestine and dogfish brain) or (ii) 20 mM sodium phosphate (pH 8.4) (porbeagle brain and dogfish intestine). The column was eluted at a flow rate of 10 ml/min with a linear gradient to (i) 1 M NaCl or (ii) 3.3 M acetic acid over 50 min. All solvents contained 10% (vol/vol) acetonitrile. Fractions were collected at 1-min intervals.

Peptide Purification. The columns were eluted with gradients of increasing concentration of acetonitrile (ranging from 1 to 0.2% (vol/vol)). Columns were used as follows: A, a 22 × 250 mm C4 column with 10-15-μM packing (Vydac); B, a 4.6 × 220 mm Aquapore phenyl (Brownlee); and a number of Vydac columns, all with 5-μM packings: 4.6 × 250 mm C4 (C) or C8 (D) and 2.1 × 150 mm C8 (E) or C18 (F). Flows of 8, 1, and 0.2 ml/min were used for the 22-, 4.6-, and 2.1-mm columns, respectively. Combinations used were Dogfish brain, C, B, E, and F; Dogfish intestine, A, C (using ammonium acetate), B, D, and E; Porbeagle brain, C, B, and E; and Porbeagle intestine, C, B, D, E, and F. Depending on the expected degree of purity, 0.5-min fractions were collected automatically or peak fractions were collected manually.

**Mass Spectrometry and Protein Sequence Analysis.** For mass spectrometry 0.5 μl of fractions containing purified peptide (in some cases concentrated ×10) were mixed with 0.5 μl of 33 mM α-cyano-4-hydroxycinnamic acid in acetonitrile/methanol (Hewlett-Packard). An 0.5-μl aliquot of this mixture was analyzed by matrix-assisted laser desorption mass spectrometry in the linear mode with both positive and negative acceleration at 20 kV using a BioFlex Instrument (Bruker–Fränzen). The method has an accuracy of 0.1%. The amino acid sequences of the purified peptides (5-100 pmol aliquots) were determined using an automated protein sequencer (Procise 494A, ABD, Perkin–Elmer). All reagents and solvents were from Applied Biosystems.

**Gene and cDNA Cloning.** Genomic DNA was isolated from dogfish spiral intestine, while total RNA was purified from the brain for isolation of CCK mRNA, and from the spiral intestine for isolation of gastrin mRNA. The procedures used for this, as well as genomic library screening, polymerase chain reaction (PCR), analysis of isolated genomic DNA, and cDNA sequencing were performed as previously described (7).

First-strand cDNA for PCR was synthesized from 5 μg of total RNA for 3′ RACE (rapid amplification of cDNA ends) and 1 μg of mRNA for 5′ RACE. The primer for the first-strand cDNA synthesis was the SGATTCG17 oligonucleotide (GATCGATGACGATCCTGACATCAGT). Isolation of the 3′ end of dogfish CCK cDNA was performed using a degenerate CCK-specific primer (SCCK3′R, Fig. 4) and the ×SC oligonucleotide (GATCGATGACGATCCTGACATC). The 5′ end of the CCK cDNA was determined using the Marathon cDNA amplification kit (CLONTECH), two nested antisense gene-specific primers (SCCK5′R1 and SCCK5′R2, Fig. 4), and the adaptor-specific primers, AP1 and AP2, included with the kit.

The dogfish gastrin cDNA was isolated by using the same procedures and primers, apart from the degenerate gastrin-specific oligonucleotide (SGAS3′R, Fig. 5) for the 3′ RACE, and two nested antisense gene-specific primers (SGAS5′R1 and SGAS5′R2, Fig. 5) for the 5′ RACE.

The genomic library was made from approximately 500 μg of dogfish DNA, and fragments in the size range 15–22 kb, generated by partial digestion with Sau3AI (Boehringer Mannheim), were ligated into BamHI-prepared arms of AGLEM11 as described (7). Escherichia coli KW251 were used as host bacteria, and 3 × 10⁶ individual phage clones were obtained and amplified as 20 independent pools.

Independent pools of the genomic library were screened by PCR with the degenerate SCCK3′R and SCCK3′ CCK-specific primers (Fig. 4), giving a 319-bp product. Two positive pools were identified from which two positive clones (ASCK2 and ASCK3) were isolated, using the 319-bp product as probe. The two phage clones contained 20 and 14 kb of genomic DNA, respectively. DNA from these recombinant phages was subjected to restriction enzyme mapping and Southern blotting and probed with radiolabeled SCCK3′ and SCCK5′T (Fig. 4). A 2.8-kb SacI fragment from both phage clones hybridized with SCCK3′, but it did not encode the 5′ end of the CCK gene, so the library was re-screened with a 3.1-kb PCR fragment obtained with the SCCK5′U and SCCK5′TA primers (Fig. 4). One positive clone (ASCK14) was isolated and subjected to restriction enzyme mapping and Southern blotting using as a probe radiolabeled SCCK5′U, SCCK5′T, and SCCK3′ oligonucleotides (Fig. 4). A 700-bp SalI fragment binding the SCCK5′U probe contained the 5′ untranslated exon 1, a 1.8-kb SacI fragment contained the 5′ end of exon 2, and a 350-bp SalI fragment encompassed the 3′ end of exon 2.

For isolation of the gastrin gene, independent pools of the genomic library were screened by PCR with SGAS5′3 and SGAS3′ gastrin-specific primers within exon 3 (Fig. 5). Two positive pools were identified and screened using the above-mentioned 279-bp PCR product as probe. One plaque containing 18 kb of genomic DNA (AGAS3) was isolated. Phage DNA from this clone was subjected to restriction enzyme mapping and Southern blotting with radiolabeled oligonucleotide probes. A 5.5-kb SacI fragment and a 5.0-kb XhoI hybridized with SGAS3′ (Fig. 5), while exon 1, exon 2, and the 5′ end of exon 3 were located in a 5-kb SacI fragment determined by hybridization with SGAS5′U, SGAS5′T, and SGAS5′3 (Fig. 5).

**RESULTS**

**Peptide Purification and Identification.** The CCK immunoreactive peptides were purified from the boiling water extracts of dogfish and porbeagle spiral intestine and brain (Fig. 2). All peptides were identified by sequence analysis (data not shown) and mass spectrometry. In addition, the mass spectrometric analyses showed that all the isolated peptides were sulfated (Table 1). All peptides reacted fully with the antisera used to monitor their purification. Hence they are C-terminally amidated.
Porbeagle brain. Two different octapeptides were isolated. The major peptide had the sequence DYYGWMDF, while 10% was identical to the intestinal gastrin-8, DYTGWMDF. Of 1.9 nmol 16% was recovered as pure peptides.

**Analytical Peptide Characterization.** The cerebrum of both species contained significant amounts of CCK-8 equivalent immunoreactivity (90 and 65 pmol/g wet weight in dogfish and porbeagle, respectively). The corresponding figures for spiral intestine were 41 and 51 pmol/g, while the stomachs contained amounts so low that purification attempts were abandoned (5 and 1 pmol/g, respectively). The consecutive acetic acid extracts of the brains contained <1% of the amounts in the boiling water extracts and were not considered further, while the acetic acid extracts of intestine contained 3–10% of the amount extracted by boiling water. Immunoreactivity was measured in individual regions of dogfish brain: cerebrum (87 pmol/g), cerebellum (including lobus olfactorius) (0.5 pmol/g), and lobus opticus (20 pmol/g).

Boiling water and acetic acid extracts of stomach and spiral intestine (divided into upper and lower half) from two specimens of dogfish were analyzed by chromatography (Fig. 3). The gel filtration profile shows three sizes of peptides coinciding with the elution positions of the purified peptides (Fig. 3A). The anion-exchange chromatography showed that all peaks were identified by both antiserum 2717 and antiserum 92128 (Fig. 3B). Since the latter is specific for sulfated CCK-like peptides, all the peptides appear to be sulfated. The extracts of the upper intestine contained primarily gastrin (Fig. 3), while the lower part contained primarily CCK in the form of CCK-8 (data not shown). The profiles obtained from the

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### Table 1. Identified peptides and their molecular masses

<table>
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<th>Peptide</th>
<th>Relative yield</th>
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<td><strong>Dogfish brain</strong></td>
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<td>CCK-8</td>
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<td><strong>Dogfish intestine</strong></td>
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<td><strong>Porbeagle brain</strong></td>
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<td><strong>Porbeagle intestine</strong></td>
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</tr>
<tr>
<td>Gastrin-17</td>
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<td>2,245.0</td>
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</table>

The relative yields of the peptides purified from each of the four tissue extracts are calculated from the semiquantitative sequence analyses. The molecular masses were obtained by mass spectrometry in the linear mode at negative polarity except for gastrin-49, which gave the best spectrum in positive mode. When measured in the positive mode all the other peptides showed exclusively molecular masses 80 Da lower, identifying loss of the labile sulfate group (20). The theoretical values were calculated from the sequences (Figs. 4 and 5), including sulfation of the Tyr residue in position 7 from the amidated C terminus.
stomach extracts resembled those from the upper intestine. Low amounts of the larger, less acidic, peptides were identified in some of the acetic acid extracts. Only one peptide, resembling CCK-8, was revealed in boiling water extracts from the brain.

Cloning of the Dogfish CCK and Gastrin Genes and cDNAs. In total the dogfish CCK mRNA was 690 nucleotides in length, encoding a 126-residue prepro-CCK. This preprohormone possesses an N-terminal hydrophobic signal sequence, as well as primary sequence requirements for the post-translational production of CCK-8 (Fig. 4). The CCK gene consists of three exons and two introns (Fig. 4). The first exon is 35 bp in length; the second exon, which possesses the ATG translation start codon and a SacI restriction enzyme recognition sequence, is 243 bp; and the third exon is 414 bp in length and contains the stop codon and AATAAA polyadenylation consensus sequence.

The dogfish gastrin mRNA was determined to be 647 nucleotides in length, encoding a 113-residue preprogastrin protein. This preprohormone contains an N-terminal hydrophobic signal sequence, as well as the sequence requirements for the post-translational production of gastrin-49 and -8, including cleavages at dibasic sites, while production of gastrin-17 requires a monobasic cleavage, violating earlier proposed consensus rules (21) (Fig. 5). The dogfish gastrin gene is 4.8 kb in length and consists of three exons and two introns (Fig. 5). The first exon is 53 bp in length; the second exon, which possesses the ATG translation start codon, is 216 bp; and the third exon is 378 bp in length and contains a SacI restriction enzyme recognition sequence, an ochre stop codon, and the AATAAA polyadenylation consensus sequence.

DISCUSSION

In the present study we have identified two separate members of the CCK/gastrin family in two shark species, the spiny

FIG. 4. Dogfish CCK gene. The three exons are capitalized and the ORF is underlined with the encoded amino acids written beneath; * indicates the translational termination codon. Position and orientation of the oligonucleotides are marked by arrows. Oligonucleotide SCCK3’R is degenerate with the following sequence: 5’-GAWTAWGTTGGAATGGCAGATT-3’, where W is A or T and I is inosine. Transcriptional regulatory elements are boxed and the putative TATA box is underlined.

FIG. 5. Dogfish gastrin gene. For explanations see Fig. 4. Oligonucleotide SGAS3’R is degenerate with the following sequence: 5’-MGIAGYTTTGACATGCA-3’, where M is C or A, Y is C or T, R is A or G, and I is inosine. The CREB transcriptional regulatory element is boxed and the TATA box is underlined.
dogfish (*Squalus acanthias*) and the porbeagle (*Lamna cornuta*). Thus the segregation of the CCK/gastrin family dates at least 350 million years back to the emergence of cartilaginous fish (22). As discussed below, we have named the two peptides CCK and gastrin. Gastrin-17 is common to the two sharks, indicating that gastrin occurred during evolution of the two shark families or at a stage earlier than their divergence.

**Gene Structures.** Both the CCK and gastrin genes, and the cDNAs they encode, were identified in the spiny dogfish. Previously, CCK and gastrin genes have been characterized from a few species (summarized in ref. 7). In each instance the genes possess a 5′ untranslated exon followed by two coding exons. The same gene structure is seen in the dogfish. In addition, several features of the dogfish genes are similar to the corresponding bullfrog genes (7). First, the three regulatory elements identified in the dogfish CCK gene (Fig. 4) are also present in the bullfrog CCK gene, showing the same order and similar spacing. Second, the intron sizes are very similar between the two species, including the large second introns of the two CCK genes (>8 kb). Third, like the bullfrog genes, exon 2 of both dogfish genes have only two nucleotides before the ATG start codon. Finally, alignment of the preprohormones indicates that the second introns are positioned at the same point of the coding sequences of both sets of genes (Fig. 6).

One notable difference between the two gastrin genes is that the dogfish promoter region contains both a consensus CREB site and a TATA box, whereas the bullfrog gene possess no obvious regulatory elements (7). Furthermore, exons 3 of the dogfish CCK and gastrin genes are similar in size (414 and 378 bp, respectively), whereas the corresponding exons of the bullfrog genes differ markedly, being 394 and 186 bp in length, respectively (7).

**Naming of the Shark Peptides.** The two dogfish prepropeptides show notable similarities (Fig. 6A), in accordance with the view that they may have evolved from a common ancestor. Both peptides also show similarities to procionin (Fig. 6B), supporting the idea that cionin is ancestral in the CCK/gastrin family (13).

One of the deduced prepropeptides shows much higher similarity to known CCKs—e.g., bullfrog CCK—than to the other dogfish prepropeptide (Fig. 6B). Furthermore, it is the major product in the brain, like CCK in mammals. Thus it appears justified to name this peptide CCK. Porbeagle CCK contains a substitution of Met to Val in position 6 from the C terminus. The same substitution is also present in guinea pig CCK (23). Hence, it appears to be a conservative substitution without functional implications. The other prepropeptide displays considerably less similarity with known CCKs but a notable similarity with bullfrog gastrin (Fig. 6B). Because it is likely to correspond to an ancestor of the nonmammalian gastrins, we name the second peptide gastrin. Additional observations support such contention: First, all CCKs, including dogfish CCK, contain two Tyr residues in the C-terminal flanking peptide, while dogfish progastrin contains only one. This region of dogfish pro-CCK is almost identical to that of the bullfrog pro-CCK and very similar to procionin (Fig. 6). Second, the gene structures of the two dogfish peptides, including sizes and positions of the introns, are very similar to those of the bullfrog counterparts.

Dogfish gastrin is primarily produced in the upper part of the intestine, while the lower part primarily produces CCK. Notably, the major production site for gastrin in tetrapods is the antral mucosa (3, 5, 25), but gastrin is also produced in duodenum and—with decreasing density—further down the intestinal tract. Furthermore, peptides (most likely identical to intestinal gastrin) are also produced in the dogfish stomach—even though in low concentration. Thus, the difference in gastrin production between sharks and “higher” animals is a matter of the relative abundance of two types of endocrine cells.

**Functional and Evolutionary Aspects.** The earliest known occurrence of acid secretion is apparently in cartilaginous fish (26). Hence it is plausible that gastrin emerged at this time in evolution as a separate acid regulatory system. However, the substitutions from CCK to gastrin in the two sharks appear moderate in the region defining biological activity (Met to Thr in dogfish, and Val to Thr in porbeagle in position 6 from the C terminus), so one might speculate whether the two shark peptides regulate separate functions, thus also justifying the names functionally in addition to the structural arguments discussed above.

CCK/gastrin receptor(s) have been characterized in several tissues of the endothermic mako shark (gallbladder, pyloric stomach, intestine, and brain) by their binding of mammalian peptides (27). In all tissues CCK and sulfated gastrin bound equally well, while nonsulfated gastrin bound 20–140 times less efficiently. Thus, the shark receptors require the peptides to be sulfated but do not distinguish between sulfation in position 7 or 6 from the C terminus. In this way they resemble the receptors in trout gallbladder (28, 29). It was suggested that the mako shark expresses a single “CCK-X” receptor, and consequently that “this species has only a single CCK regulatory peptide, rather than separate CCK and gastrin peptides” (27). The mako shark and porbeagle belong to the same family (the Lamniformes), while the dogfish belong to the Squatiformes (30). Since a separate gastrin peptide is found in both dogfish and porbeagle, a gastrin is also likely to occur in mako shark. If the mako shark (and other Chondrichthyes) have two receptors distinguishing the subtle differences of the two peptides, studies as described above are unlikely to identify them.

Recent experiments have demonstrated that bullfrog tissues distinguish the moderate structural differences of the endogenous gastrin and CCK, with Ala and Met, respectively, in position 6 from the C terminus (10). Thus, bullfrog CCK and gastrin constitute separate peptide systems in spite of their close resemblance in the region crucial for biological activity. Hence, it appears likely that this is also the case in the sharks, but experiments similar to those performed in the bullfrog and receptor binding studies using the authentic shark peptides, will be needed to determine if such a functional segregation has indeed occurred already at the elasmobranch level.

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**Fig. 6.** Comparison of dogfish CCK and gastrin prepropeptide sequences with cionin (**A**) and bullfrog CCK and gastrin (**B**). The data were generated by using the *gap* alignment program (31) of the GCG software package (32). Identical amino acids (,), functionally conservative substitutions (.), and substitutions that could result from a single base change (.) are indicated. The box in **B** indicates the position of intron 2, which in all four genes divides the first and second base of the codons for the respective framed amino acid residues. The sources of sequences are ref. 33 for cionin and ref. 7 for bullfrog gastrin and CCK.
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