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Human pancreatic icosapeptide: Isolation, sequence, and immunocytochemical localization of the COOH-terminal fragment of the pancreatic polypeptide precursor

(evolution/protein chemistry/post-translational modification/peptide colocalization)

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ABSTRACT **In dogs, the COOH-terminal part of the pancreatic polypeptide precursor gives rise to a stable icosapeptide product against which an antiserum has been raised. By immunohistochemistry, icosapeptide immunoreactivity was localized in human pancreas exclusively to cells that also stored pancreatic polypeptide. Analytical peptide chemistry demonstrated that a peptide corresponding to the canine icosapeptide could be extracted from the pancreatic polypeptide-rich duodenal part of the human pancreas. The human pancreatic icosapeptide was isolated by acid ethanol extraction, gel filtration, anion-exchange chromatography, and reverse-phase high-performance liquid chromatography. The COOH-terminal sequence of the human icosapeptide is very similar to that of the canine icosapeptide, whereas none of the first nine amino acid residues are identical. When the primary structure of peptides from three different species are compared, it is apparent that the pancreatic polypeptide part of the common precursor is a well-conserved sequence as compared to the icosapeptide part, although 8 out of 11 residues in the COOH-terminal sequence of the icosapeptide are identical in all three species.**

Peptide hormones are generated through post-translational modification of larger precursor molecules; this processing can result in the formation of several stable peptide products of which more than one might function as a messenger molecule (1). It is generally believed that the structure of a biologically important part of a precursor is better preserved through evolution than a peptide fragment with no postsecretory action. This has been extensively studied in proinsulin, where the primary structure of the C peptide, which during the biosynthesis connects the A and B chains of the insulin molecule, varies considerably between species as compared to the structure of the biologically active peptide, insulin (2).

The hormone pancreatic polypeptide (PP) (M_r 4,250) is synthesized as the NH_2-terminal part of a precursor with a M_r of 8,000–10,000 (3). In dogs we have studied the biogenesis and structure of an icosapeptide that is a stable peptide product excised from the COOH-terminal part of the PP precursor as a COOH-terminally extended intermediate form (3, 4). In the present paper, we present immunohistochemical evidence that a homologous peptide to the dog icosapeptide is stored in human PP cells and chemical evidence that the sequence of the human icosapeptide is not as conserved as the primary structure of the PP molecule.

MATERIALS AND METHODS

Immunocytochemistry. Specimens of human pancreas were obtained at surgery from three patients; the resection was performed to remove nonendocrine tumors (two patients) or an insulinoma (one patient). The specimens were rapidly frozen to the temperature of liquid nitrogen in a mixture of propane and propylene and freeze-dried. They then were vapor-fixed in formaldehyde for 1 hr at 80°C and embedded in paraffin or Araldite (5). Sections were cut at 6-μm (paraffin) or 2- to 3-μm (plastic) thickness. After removal of the embedding medium, the sections were hydrated and processed for the immunocytochemical demonstration of PP or pancreatic icosapeptide by the indirect immunofluorescence procedure of Coons *et al.* (6) or the immunoperoxidase procedure of Sternberger (7). Sequential immunostaining of one and the same section for PP and icosapeptide was performed by the technique of Tramu *et al.* (8). The PP antiserum (code no. 249, raised against human PP; donated by R. E. Chance of Eli Lilly Research Laboratories, Indianapolis, IN) was used at 1:320 dilution (immunofluorescence) or 1:1,280 dilution (immunoperoxidase). The icosapeptide antiserum (code no. 3204, raised against caninepancreatic icosapeptide) was used at 1:80 dilution (immunofluorescence) or 1:320 dilution (immunoperoxidase). Sections incubated with peptide antiserum exposed to excess amounts of antigen (10–100 μg of human PP or canine icosapeptide per ml of diluted antiserum) served as controls.

Peptide Isolation. Specimens of human donor pancreas were obtained through the National Diabetes Research Interchange. The pancreatic tissue had been separated into three pieces—head, body, and tail—and frozen on dry ice. For preparative purpose, 285 g of heads of pancreas were homogenized in a Waring Blendor in acid ethanol at −20°C [0.1 mol of HCl per liter of ethanol (final concentration, 68%)]. The homogenization was repeated several times; when the temperature reached 4°C, the homogenate was set to incubate overnight at this temperature. The homogenate was then centrifuged at 10,000 × *g* for 20 min at 4°C; the supernatant was neutralized with an aqueous ammonia solution and was centrifuged at 2,000 × *g* for 5 min at 4°C. The supernatant was then supplemented with 2 vol of ice-cold absolute ethanol and 4 vol of ice-cold ethyl ether. The peptide precipitate, which formed through incubation overnight at 4°C, was dried over nitrogen and dissolved in acetic acid (3 mol/liter) and gel-filtered on a 5 × 180 cm Bio-Gel P-30 (Bio-Rad) column with acetic acid (3 mol/liter) as eluant.

The fractions from the gel filtration column containing the icosapeptide [identified by polyacrylamide gel electrophoresis (15% acrylamide, pH 8.7); ref. 9] were pooled, dried down, and reconstituted in Tris (0.02 mol/liter), adjusted to pH 8.5 with HCl, containing urea (Schwarz/Mann) at 6.5

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Abbreviation: PP, pancreatic polypeptide.

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mol/liter, and the peptides were purified further on a 1 × 5 cm DEAE-Sephadex (Pharmacia) ion-exchange column, which was equilibrated and eluted at 4°C in the Tris buffer with a NaCl gradient of 0–0.15 mol/liter (total volume of gradient, 500 ml). The fractions that contained the icosapeptide were pooled and dialyzed at 4°C against acetic acid (1 mol/liter) in Spectrapor-3 dialysis tubing (Spectrum Medical Industries, Los Angeles) which had been pretreated by boiling for 5 min in EDTA in water (0.01 mol/liter) brought to pH 8 with NaOH. The peptides were finally purified on a Nucleosil 10 C_{18} reverse-phase column (8 × 240 mm; Macherey & Nagel), which was eluted at 50°C with 0.1% trifluoroacetic acid (Pierce) in water containing a 10–60% gradient of acetonitrile (HPLC grade; Rathburn, Walkerburn, Scotland). The chromatography was performed on a Hewlett–Packard 1084 B liquid chromatograph. After both dialysis and liquid chromatography, the solvents were removed by vacuum desiccation.

For analytical purposes, one head and one body of human pancreas were extracted separately as described above, and peptides were separated by gel filtration on a Bio-Gel P-30 column (2.5 × 95 cm) eluted with acetic acid (3 mol/liter). Aliquots from fractions containing peptides with an apparent M_r of 1,000–8,000 were characterized on polyacrylamide slab gel electrophoresis (10% acrylamide, pH 8.7; ref. 9), which was stained by amido black.

Peptide sequence determination was performed on a Beckman 890C spinning cup sequencer (generously put at our disposal by S. Magnusson, Department of Molecular Biology, University of Aarhus, Denmark) with chemicals and programming as recommended by the manufacturer. Aminoacylphenylthiohydantoin derivatives were identified on a reverse-phase system by using a Spherisorb S5 ODS2 (Phase Sep, Queensferry, Clwyd, U.K.) column and an ethanol gradient system on a Hewlett–Packard 1084 B liquid chromatograph (10). The yield of aminoacylphenylthiohydantoin derivative in cycle 1 was 60 nmol. The COOH-terminal sequence of the peptide was studied by degradation with carboxypeptidase Y (a generous gift from J. Johansen, Carlbiotech, Copenhagen) at 37°C on 2 nmol of peptide with a molar enzyme/peptide ratio of 1:50 in *N*-ethylmorpholine (0.1 mol/liter) brought to pH 7.0 with acetic acid. Norleucine (5 nmol) was used for internal standardization, and aliquots were removed after 0, 1, 5, 15, and 25 min of incubation. Free amino acid content was determined on an LKB 4400 amino acid analyzer.

RESULTS

Gel filtration and polyacrylamide slab gel electrophoresis of peptides extracted from human pancreas indicated that a peptide corresponding to the pancreatic icosapeptide previously isolated from dog pancreas was found in the head of the pancreas (Fig. 1). The size and electrophoretic mobility of the human and canine peptides were indistinguishable. Only trace amounts of this peptide could be detected in the body of the pancreas (Fig. 1).

Immunocytochemical staining, double staining, or staining of consecutive plastic sections with antibodies directed against PP and the canine icosapeptide showed a colocalization of PP and icosapeptide immunoreactivity in islet cells as well as in single scattered cells in the acinar tissue or in the duct epithelium (Fig. 2). Whereas icosapeptide immunoreactivity was found exclusively in PP immunoreactive cells, a minor population of these cells (approximately 10%) did not stain with the icosapeptide antiserum.

The peptide identified as the human counterpart to the canine pancreatic icosapeptide was purified from human duodenal pancreas by extraction with acid ethanol, precipitation with ether, anion-exchange chromatography, and reverse-phase liquid chromatography as shown in Fig. 3. The purification was monitored by analytical polyacrylamide gel electrophoresis. During the anion-exchange chromatography, the desired peptide was eluted both in the break-through together with glucagon and in a distinct peak during the isocratic period of elution (Fig. 3*B*). The icosapeptides from both protein peaks were finally purified by the reverse-phase liquid chromatography and were pooled (Fig. 3*C*).

Edman degradation of the peptide gave a clear identification of aminoacylphenylthiohydantoin derivatives in the first 19 steps, with the yield decreasing from 60 to 8 nmol of derivative (Fig. 4). No aminoacylphenylthiohydantoin derivative was identified after the 19th cycle of Edman degradation. Carboxypeptidase Y digestion of the peptide demonstrated that after 1 min of incubation, 0.91 residue of arginine, 0.81 residue of proline, and 0.46 residue of valine were released; after 5 min of incubation, 1.00 residue of valine and 1.54 residues of alanine were released. These data indicate that the COOH-terminal residue of the icosapeptide is arginine (Fig. 4). As in the canine peptide, the carboxypeptidase had difficulty in removing the histidine-16 carboxyl-terminal to the proline-15 residue, as only 0.12 and 0.31 residue of histidine, respectively, was released after 5 and 15 min.

Only 50% of the amino acid residues are identical between the canine and the human icosapeptides (Fig. 5). However,

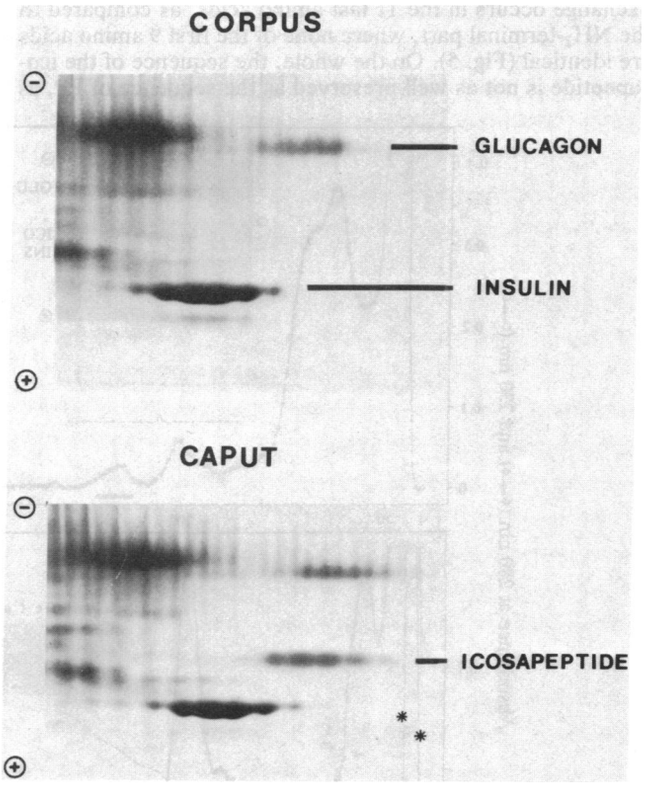

FIG. 1. Amido black-stained peptides after polyacrylamide slab gel electrophoresis of aliquots from gel filtration fractions of acid ethanol extracts of human pancreas. (*Upper*) Peptide pattern from the body of the pancreas (corpus) showing the positions of glucagon and insulin. (*Lower*) Peptide pattern from the duodenal part or the head of the pancreas (caput) showing the position of the icosapeptide. The positions of two additional peptides which, like the icosapeptide, are found almost exclusively in the head of the pancreas are indicated by asterisks. Gel filtration was performed on Bio-Gel P-30 columns, and aliquots from fractions of apparent M_r ≈8,000–1,000 were characterized in gels containing 10% acrylamide (pH 8.7). The human pancreatic polypeptide, which on gel filtration almost coelutes with insulin, does not migrate into these gels because of its high isoelectric point.

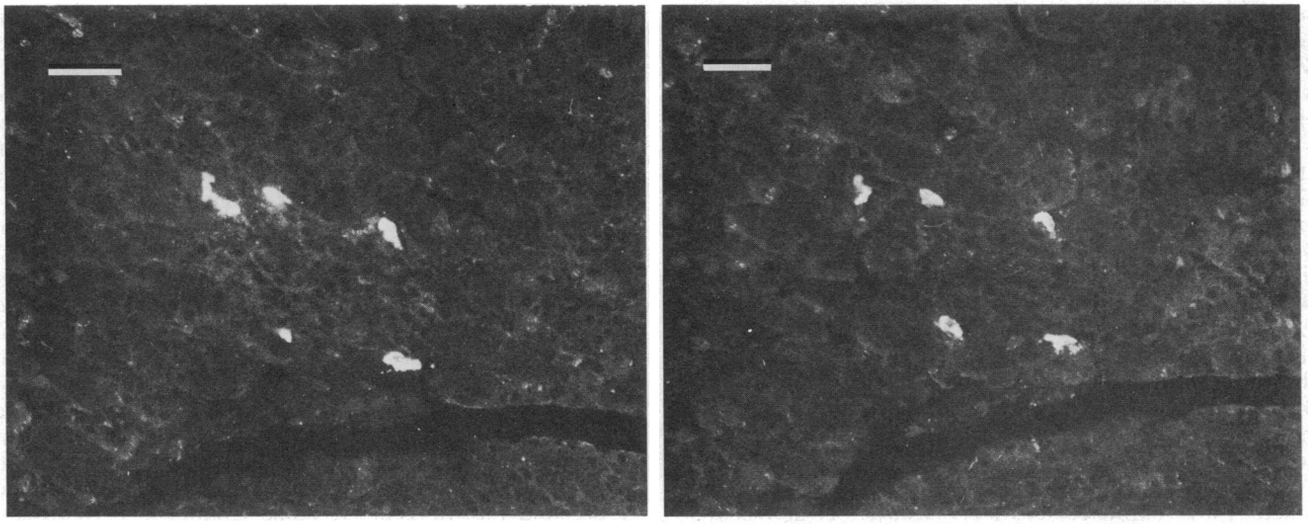

FIG. 2. Immunohistochemical localization of PP and pancreatic icosapeptide. Consecutive plastic sections showing icosapeptide immunofluorescence (*Right*) and PP immunofluorescence (*Left*) in cells located peripherally in an islet in human pancreas. Note that the PP immunofluorescent cells are identical with those displaying icosapeptide immuofluorescence. (×200.) (Bar = 50 μm.)

there is a remarkable difference between how well the COOH-terminal part of the molecule is preserved—only 1 exchange occurs in the 11 last amino acids, as compared to the NH_2-terminal part, where none of the first 9 amino acids are identical (Fig. 5). On the whole, the sequence of the icosapeptide is not as well preserved as the sequence of PP, as there are only 2 amino acids out of 36 that differ between the canine and the human PP (Fig. 5).

DISCUSSION

In the present paper, the amino acid sequence and immunocytochemical localization of the human pancreatic icosapep-

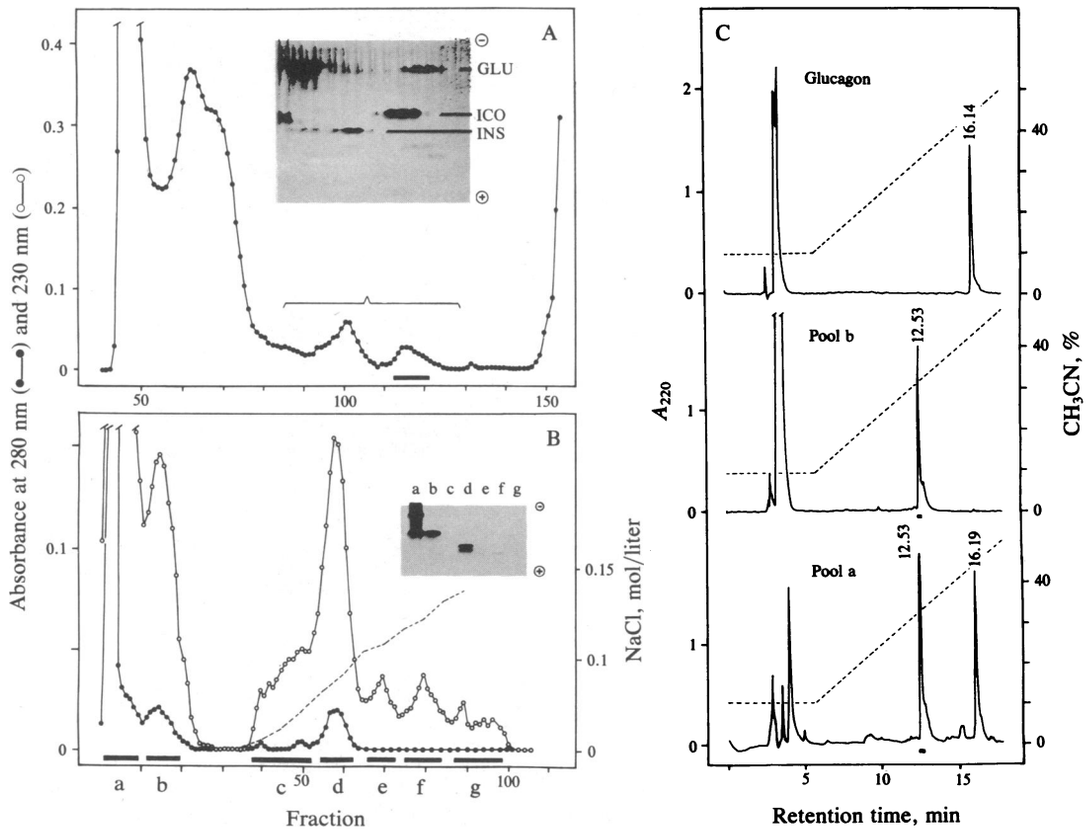

FIG. 3. Purification of the human icosapeptide. (*A*) Gel filtration profile (Bio-Gel P-30) of proteins extracted from 285 g of human duodenal pancreas. (*Inset*) Polyacrylamide slab gel electrophoresis of aliquots from selected fractions as shown; the positions taken by glucagon (GLU), icosapeptide (ICO), and insulin (INS) are marked. The solid bar indicates the fractions that were pooled and evaporated for further purification. (*B*) Elution profile of gel-filtered peptides on DEAE-Sephadex. ---, NaCl gradient. (*Inset*) Polyacrylamide slab gel electrophoresis of aliquots from the different pools of peptides. Pools a and b were purified further. (*C*) Elution profile of peptides on reverse-phase HPLC of the two pools a and b from ion-exchange chromatography and of a glucagon standard. ---, Acetonitrile gradient. The solid bars indicate the peak material collected for structural analysis.

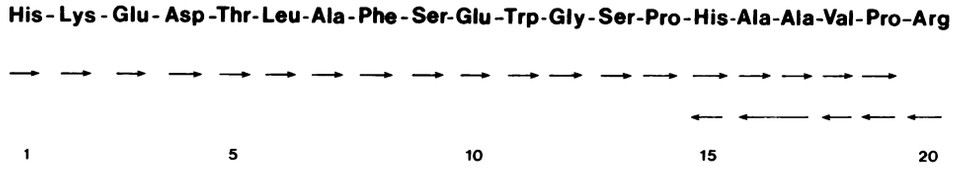

FIG. 4. Amino acid sequence of the human pancreatic icosapeptide. The amino acid residues that were placed in sequence by sequential Edman degradation are indicated by arrows pointing right. The amino acid residues that could be placed in sequence by degradation with carboxypeptidase Y are indicated by arrows pointing left.

tide are presented. Previously, we have studied the biogenesis and structure of the canine pancreatic icosapeptide (3, 4). At present, the following evidence indicates that the pancreatic icosapeptide is a second stable product from the PP precursor. First, peptide mapping of biosynthetically labeled peptides indicates that the PP precursor and the icosapeptide share identical tryptic fragments (3). Second, the icosapeptide and PP show a parallel rate of appearance during pulse–chase experiments examining precursor processing (3). Third, the PP precursor can be immunoprecipitated specifically by antibodies raised against the icosapeptide (4). Finally, icosapeptide immunoreactivity is found exclusively in PP-storing cells (Fig. 2).

A few cells in the human pancreas reacted only with the PP antiserum and not with the icosapeptide antiserum. These could represent cells in which the amount of PP, and thereby also icosapeptide, is too low to be detected by the icosapeptide antiserum raised against the canine icosapeptide. Note that the antiserum might react only weakly with the somewhat different human icosapeptide. This is likely because another antiserum that works perfectly well in immunocytochemistry on canine pancreas does not detect the human icosapeptide (unpublished observation). Another possibility would be that some PP immunoreactive cells could store another peptide from the PP peptide family. This family consists at present of PP (11), peptide YY (12), and neuropeptide Y (13). In the distal intestine, peptide YY-immunoreactive cells stain with certain PP antisera, but not with icosapeptide antisera; and the ileum and colon do not contain any extractable PP immunoreactivity (unpublished observation). In the present context, the most important observation is, however, that the icosapeptide is found only in cells that also react with PP antisera (Fig. 2).

To present a more complete picture of the variability between species of the different parts of the common PP-icosapeptide precursor, we have included in Fig. 5 the sequence of the recently isolated ovine icosapeptide (14) and that of the corresponding ovine PP (11). The PP sequences vary only at four scattered positions in the NH_2-terminal part of the 36-amino-acid molecule; whereas the three icosapeptides differ at 12 out of the 20 positions. When all three different icosapeptides are considered, it is still apparent that the COOH-terminal end of the molecule is the best-preserved part (Fig. 5). The icosapeptide is excised from the precursor as an intermediate form that bears five additional amino acids, none of which are basic, as a COOH-terminal extension (4, 15). The COOH-terminal amino acid residues of the icosapeptide, proline-19 and arginine-20, that preceed this monobasic cleavage site are preserved in all three species. A monobasic cleavage site is found in a few other precursors, such as the common precursor for vasopressin–neurophysin-II (16) and presumably also in the precursor for cholecystokinin-8, if the cholecystokinin-33 is an intermediate form derived from the same precursor (17).

The original incentive to search for another peptide product from the PP cell was the observation that the PP cell was more sensitive to small decrements in blood glucose than, for example, the glucagon cell (4, 18). The only known product from the PP cell, PP itself, was without metabolic actions in mammals (19, 20), although the peptide was a well-established regulator of zymogen secretion from the exocrine pancreas and gall bladder motility (21). In preliminary experiments, the icosapeptide also lacked metabolic effects as seen, for instance, on plasma concentrations of glucose, free fatty acids, insulin, and glucagon when the canine icosapeptide was infused into dogs in reasonable amounts (unpublished observations). Although it is still possible that the icosapeptide might have some postsecretory action, the fact that its amino acid sequence is so variable among species could indicate that this part of the PP precursor has not been subjected to evolutionary structure-functional conservation. Nevertheless we do know peptide hormones—e.g., somatotropin and calcitonin—that vary considerably, even among mammalian species (2).

Note Added in Proof. Molecular cloning of a human PP cDNA, performed in collaboration with E. Boel and co-workers at the Laboratory of Genetics, NOVO Research Institute, Denmark, has recently confirmed both the general structure of the PP precursor and the amino acid sequence of the human pancreatic icosapeptide as presented in this paper.

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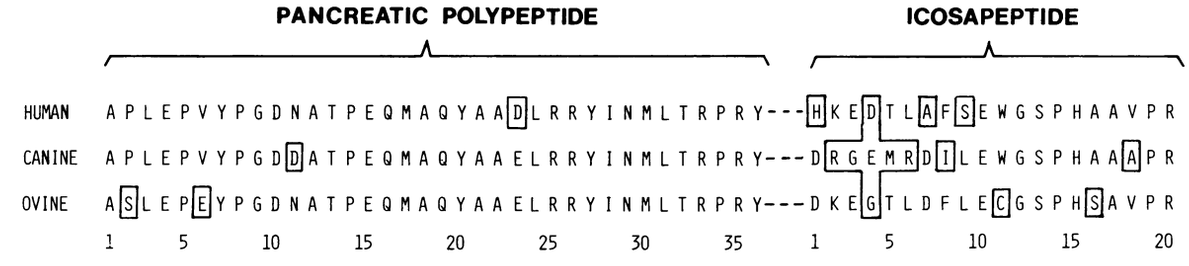

FIG. 5. Amino acid sequence of the human, canine, and ovine PP and pancreatic icosapeptides. – – –, Intervening sequence between the PP and the icosapeptide in the common precursor. Amino acid residues that differ among the three species are indicated by boxes. The COOH-terminal tyrosine residue in PP is α-carboxyamidated. In the dog, the icosapeptide is known to be excised from the precursor as an intermediate form bearing a COOH-terminal extention of five amino acids.

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