Isolation and primary structure of tumor-derived peptides related to human pancreastatin and chromogranin A

(amino acid amide/posttranslational processing/carcinoid tumor/radioimmunoassay/immunocytochemistry)

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ABSTRACT Using an antiserum raised against a synthetic C-terminal peptide of porcine pancreastatin, we detected pancreastatin-like immunoreactivity (PLI) in human pancreatic islets, adrenal medulla, and endocrine tumors. From a carcinoid liver metastasis, human PLI was extracted and purified by HPLC. Two C-terminally amidated peptides were isolated and characterized by sequence analysis. The first peptide, hCgA-210–301, consists of 92 amino acid residues with glycine as C terminus. It is identical to the cDNA-derived sequence of human chromogranin A, positions 210–301, which is preceded by two basic residues indicating a putative processing site. The C-terminal part, positions 250–301, shows 70% sequence identity to porcine pancreastatin and represents the human pancreastatin-like sequence. The second peptide, hCgA-273–301, represents a C-terminally amidated fragment of the human pancreastatin sequence, generated by an Asp-Pro cleavage at the N terminus. Peptide hCgA-273–301 was synthesized to confirm the structure of the natural peptide. Two other peptides derived from human chromogranin A were isolated and partially characterized. They are generated by proteolytic cleavage after dibasic amino acids Lys-Arg (positions 338–339) and after Trp-376 of the human chromogranin A sequence, respectively. These results indicate that chromogranin A may represent the precursor for pancreastatin-related and possibly other yet-unidentified peptides of unknown physiological function.

Pancreastatin, a peptide comprising 49 amino acid residues, was isolated from porcine pancreas by Tatemoto *et al.* (1), using its C-terminal glycine as isolation criterion. Porcine pancreastatin shows striking sequence homology to a part of bovine (2–6), human (7), and rat chromogranin A (CgA) (8), a protein present in the secretory granules of endocrine and neuronal cells (9). Recently, it has been shown that porcine CgA is the pancreastatin precursor (10). The presence of several paired basic amino acid residues in the sequence of human, bovine, and rat CgA could indicate that proteolytic processing to other, yet-unidentified, regulatory peptides may occur. It has been reported that porcine pancreastatin-(1–49) as well as its C-terminal fragments pancreastatin-(33–49) and -(29–49) inhibits insulin secretion in rats (1, 11–13), but the widespread occurrence of pancreastatin-like peptides and CgA in a variety of endocrine and neuroendocrine cells indicates that the biological function of these peptides may not be restricted to the pancreas.

To investigate the relationship between CgA and pancreastatin in humans and to define the physiological role of pancreastatin as a putative regulatory peptide in various endocrine disorders—i.e., diabetes mellitus—it is of interest to determine the primary structure of the human peptide. Using an antiserum raised against the C-terminal synthetic

fragment porcine pancreastatin-(33–49) (pP-33–49), we characterized pancreastatin-like immunoreactivity (PLI) in human tissues and endocrine tumors (14). This study describes the structural characterization of peptides isolated from a human carcinoid tumor metastasis that are derived from human CgA. Two C-terminally amidated peptides are related to the putative human pancreastatin sequence.

MATERIAL AND METHODS

Peptide Synthesis. pP-33–49, the human pancreastatin fragment corresponding to human CgA residues 273–301 (hCgA-273–301), and human CgA fragment hCgA-210–225 were synthesized by using *N*^α-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid esters in an automatic solid-phase synthesizer (LKB Biolynx) (15, 16) and purified by gel filtration and preparative HPLC. C-terminal glycine was identified by an HPLC amino acid amide assay (17).

Pancreastatin Radioimmunoassay and Immunocytochemistry. C-terminal-specific rabbit antiserum pP-33–49-C8 was obtained by immunization with pP-33–49 coupled to bovine serum albumin with carbodiimide and used in a specific RIA as described (14). The antiserum recognized high molecular weight forms of PLI, indistinguishable from CgA in molecular weight or larger than it. No cross-reactivity exceeding 0.01% was observed with standard gastrointestinal regulatory peptides.

Sections (5 μ m) of Bouin-fixed and paraffin-embedded tissue were stained with antiserum pP-33–49-Glu13 (dilution 1:2000–1:5000) by using the peroxidase–anti-peroxidase method (18).

Isolation of Human CgA-Derived Peptides. Human PLI was detected by RIA using antiserum pP-33–49-C8 in an extract of a huge liver metastasis derived from a carcinoid tumor. Tumor tissue was obtained after right-sided hepatectomy and frozen immediately in liquid nitrogen. A 35-g portion of frozen tumor was dissected, boiled in water for 10 min, and homogenized (Ultra-Turrax tissue homogenizer) for two 15-sec intervals at 4°C. The extract was adjusted to 1 M acetic acid, stirred for 4 hr at 4°C, and centrifuged for 45 min at 16,000 $\times$ *g*. The supernatant was filtered through nylon gauze and passed through 16 Sep-Pak C₁₈ cartridges in series. Peptide material was eluted with 80 ml of 0.1% trifluoroacetic acid/80% acetonitrile/19.9% water (vol/vol) and concentrated by evaporation, avoiding dryness. The eluate was separated by HPLC on a preparative reverse-phase C₁₈ column [Nucleosil 300-7 wide pore (Macherey & Nagel, Düren, F.R.G.) 1 $\times$ 25 cm, flow 2.5 ml/min] using a Waters HPLC system. The solvent system consisted of 0.1% trifluo-

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Abbreviations: CgA, chromogranin A; hCgA-273–301, human CgA residues 273–301 (other fragments are abbreviated similarly); PLI, pancreastatin-like immunoreactivity; pP-33–49, porcine pancreastatin residues 33–49.

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FIG. 1. HPLC elution profile of the carcinoid tumor extract on a preparative C_{18} column in the trifluoroacetic acid/acetonitrile system; flow rate was 2.5 ml/min. Material was eluted with a linear gradient (right ordinate): 0% to 10% solvent B in 0.5 min, 10% to 70% solvent B in 120 min. Fractions (2.5 ml) were collected and assayed for PLI by using antiserum pP-33-49-C8; results are shown in the inner histogram in terms of nmol of PLI per fraction.

roacetic acid in water (solvent A) and 0.1% trifluoroacetic acid/29.9% water/70% acetonitrile (solvent B). Fractions (2.5 ml) were collected and assayed for PLI. Peak fractions were rechromatographed on an analytical wide-pore C_{18} column (Vydac RP218TP54, The Separations Group, Hesperia, CA; 0.46×25 cm, flow 1.5 ml/min) by using the same solvent system or 5 mM potassium phosphate, pH 6 (solvent A), and 30% solvent A/70% acetonitrile (solvent B).

Some peptides were cleaved with trypsin (1:40, wt/wt) in NH_4HCO_3 , pH 8.3, at 37°C for 24 hr. Tryptic peptides were separated by HPLC as described. The primary structures of purified peptides were determined by automatic Edman degradation on a gas-phase sequencer (470A, Applied Biosystems, Foster City, CA). The C-terminally amidated amino acid residue was identified after tryptic cleavage and HPLC separation as the phenylthiocarbamoyl (Ptc) derivative by an HPLC amide assay, as described (17).

RESULTS

Isolation and Characterization of Human CgA-Derived Peptides. The initial homogenate of the liver metastasis from a human carcinoid tumor contained 5.9 nmol of PLI per g of tumor, as measured by RIA with C-terminal-specific pancreastatin antiserum pP-33-49-C8. The extract was passed through Sep-Pak cartridges and separated by HPLC on a preparative reverse phase C_{18} column (Fig. 1). Human PLI was eluted in two major peaks at 59 and 62 min; porcine pancreastatin injected for calibration was eluted at 60 min. Total recoveries from six identical HPLC runs were 14 nmol

for peak I (PI) and 26 nmol for peak II (PII). Fractions containing these two peaks were combined and partially evaporated. Further purification was achieved on an analytical reverse-phase C_{18} wide-pore column using the trifluoroacetic acid/acetonitrile solvent system. Rechromatography of peak I yielded a single homogenous immunoreactive fraction, PF1 (Fig. 2). Peak II could be resolved by HPLC rechromatography into two PLI-containing fractions, PF2 and PF3 (Fig. 2). Gas-phase sequencing of these peptides and their major tryptic fragments and C-terminal analysis established the existence and structural characteristics of two C-terminally amidated peptides.

Fraction PF1 contained the C-terminal human pancreastatin fragment hCgA-273-301, which, using 2 nmol, we sequenced completely except for the ultimate residue Gly in position 301 (Fig. 3). It shows high structural homology to porcine pancreastatin and the corresponding sequences in bovine and rat CgA (Fig. 4). To investigate whether this peptide is C-terminally α -amidated, approximately 1.5 nmol were cleaved with trypsin to release C-terminal glycnamide. The tryptic mixture was separated on a C_{18} wide-pore column that had been calibrated with glycnamide as standard (Fig. 5). Care was taken not to cross-contaminate successive runs by washing the injection valve thoroughly and running cleaning-up gradients. Fractions that were coeluted with glycnamide (2.0–3.0 min) were collected and the solvent was evaporated. Coupling to phenylisothiocyanate allowed the identification of glycnamide as the phenylthiocarbamoyl derivative according to the HPLC amino acid amide assay described (17). The later-eluting peak represents peptide

FIG. 2. HPLC rechromatography of fraction PI (Left) and fraction PII (Right) on an analytical C_{18} column using the trifluoroacetic acid/acetonitrile system; flow rate 1.5 ml/min; absorbance was monitored at 214 nm (upper or only trace) and 280 nm (lower trace in Left). Material was eluted with linear gradients, as indicated with the % B scales. Fractions PF1, PF2, and PF3 contained PLI (inner histograms; nmol of PLI per fraction).

hCgA-210/301

FIG. 3. Primary structures of human CgA-derived peptides. Right-pointing arrows indicate residues identified by Edman degradation. The C-terminally amidated peptides hCgA-210-301 (92 residues) and hCgA-273-301 (29 residues) contain the human pancreastatin-like sequence. C-terminal glycine released after trypsin cleavage (left-pointing arrow) was identified by HPLC amino acid amide assay (17). Two other peptides, hCgA-P2 and hCgA-P3, are derived from the C-terminal part of human CgA.

hCgA-273-300, which contained a Lys-Glu bond and a Lys-Gly bond apparently resistant to tryptic cleavage.

Fractions PF2 and PF3, also immunoreactive with C-terminal-specific pancreastatin antiserum pP-33-49-C8, contained a larger CgA-derived peptide, which was purified to homogeneity by two successive HPLC rechromatography steps (Fig. 6) using the potassium phosphate and trifluoroacetic acid solvent systems. N-terminal sequence analysis (two runs with 70 and 350 pmol, respectively) revealed the first 29 amino acid residues (Fig. 3) except for some ambiguity at position 24 (Thr). This sequence is identical to human CgA, positions 210-238, which is located N-terminally from the putative human pancreastatin sequence (Fig. 4). Tryptic cleavage of PF3 followed by HPLC amino acid amide assay proved the existence of glycine eluting at the same position, as shown for PF1 (Fig. 5). The large peak eluting at 42 min represents the tryptic peptide hCgA-254-300, which contains 47 residues, thus confirming that the CgA-derived peptide hCgA-210-301 contains the human pancreastatin-like sequence as C-terminal part. The potential tryptic cleavage site at Lys-Gly (276-277) remained intact in the 92-residue peptide hCgA-210-301 after prolonged tryptic digestion. The primary structure of the N-terminal 26 amino acids of the tryptic peptide hCgA-254-300 (360 pmol) provided a sequence of 7 residues overlapping with the C-terminal fragment hCgA-273-301, which was eluted in fraction PF1 (Fig. 2). Thus, the complete primary structure of the C-terminally

FIG. 5. HPLC-elution profile of the tryptic peptides of fractions PF1 (Upper) and PF3 (Lower) obtained from the separation shown in Fig. 2; the fraction marked G-NH₂ contained glycine identified by HPLC amino acid amide assay. Peptides hCgA-254-300 and hCgA-273-300 were characterized by amino acid sequence analysis.

amidated tryptic peptide hCgA-254-301 was elucidated (Fig. 3); it represents the human pancreastatin-like sequence.

Two other human CgA-derived peptides were identified in the HPLC rechromatography of peptide hCgA-210-301 (Fig. 6) and partially characterized by gas-phase sequencing (Fig. 3): the N-terminal part of peptide hCgA-P2 (30 pmol) corresponds to human CgA, positions 340-356, and is derived from the parent molecule by cleavage after the two dibasic residues Lys-Arg (338-339). The N-terminal sequence of peptide hCgA-P3 (750 pmol) is identical to position 377-392 of human CgA. The N terminus is located two residues from the basic pair Arg-Arg (374-375) (Fig. 4), which could indicate processing at this site followed by N-terminal degradation of peptide hCgA-P3 by the action of a dipeptidyl aminopeptidase-like enzyme.

FIG. 4. Comparison of human, bovine, rat, and porcine CgA regions containing the pancreastatin-like sequence. hCgA, human CgA, positions 208-401 (7); bCgA, bovine CgA (3); rCgA, rat CgA (8); pCgA, porcine CgA (10); pP, porcine pancreastatin (1); x, unspecified; *, C-terminal glycine. Residues identical to human CgA are indicated by dashes. Dots represent spaces introduced for best sequence alignment. Arginine (R) and lysine (K) residues in human CgA are underlined. Bars indicate human CgA-derived peptides and porcine pancreastatin.

FIG. 6. HPLC-elution profile of fraction PF3 (*Left*) rechromatographed on an analytical C_{18} column by using the potassium phosphate solvent system; flow, 1.5 ml/min. Peaks 1 and 3 were rechromatographed in trifluoroacetic acid/acetonitrile (*Center and Right*). Peak 1 corresponds to peptide hCgA-210–301, peak 2 represents peptide hCgA-P2, and peak 3 contains hCgA-P3.

Synthesis of Human CgA-Derived Peptides. Two peptides related to human CgA and pancreastatin were synthesized by fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis and purified by HPLC. The C-terminally amidated synthetic peptide hCgA-273–301 was coeluted with the corresponding tumor-derived peptide under different gradient conditions and showed identical radioimmunological behavior, thus confirming the primary structure of this C-terminally amidated human pancreastatin fragment. To prove that pancreastatin antiserum pP-33–49-C8 recognizes the C-terminal human pancreastatin-like structure in hCgA-210–301 and does not cross-react with a similar negatively charged sequence at the N terminus of hCgA-210–301, the fragment hCgA-210–225 was synthesized. This peptide cross-reacted less than 0.01% with antiserum pP-33–49-C8 when tested in serial dilutions against porcine pancreastatin as standard.

Immunocytochemical Localization of Human PLI. When antiserum pP-33–49-Glu13 was used, endocrine cells were stained in different tissues, such as human pancreatic islets, adrenal medulla, and several endocrine tumors. In islets, pericapillary cells, most probably representing A and D cells, reacted stronger with the pancreastatin antiserum than B cells (Fig. 7A), but the pattern was variable with regard to the intensity of staining. Chromaffin cells of human adrenal medulla showed a strong positive reaction (Fig. 7B), indistinguishable from staining with a monoclonal CgA antibody (results not shown). Fig. 7C represents the liver metastasis of the carcinoid tumor from which human pancreastatin has been isolated and structurally characterized. The majority of tumor cells contain PLI.

DISCUSSION

The immunocytochemical localization of PLI resembles closely the distribution of CgA (9, 19–22). In human pancreatic islets, we located PLI predominantly in pericapillary endocrine cells representing areas of non-B cells (14). Using a C-terminal-specific antiserum, we isolated from a carcinoid tumor peptides related to human pancreastatin and CgA. Glycinamide, identified by an HPLC amino acid amide assay (17), was found to constitute the C terminus of two pancreastatin-like peptides.

hCgA-273–301 consists of 29 amino acids and is identical to the cDNA-derived sequence of human CgA, positions 273–301 (7). A similar fragment missing a Glu residue at position 290 has been isolated from a human glucagonoma (23). It remains to be shown whether this structural difference is due to a sequencing error or represents a true polymorphism in the human CgA gene. Since the N-terminal Pro residue of hCgA-273–301 is preceded by Asp in the human

CgA sequence, it has to be assumed that the acid-labile peptide bond Asp-Pro was cleaved during the purification procedure. Hitherto, no physiological *in vivo* processing at these residues has been described.

The second peptide identified, hCgA-210–301, consists of 92 amino acid residues with C-terminal glycinamide and carries the human pancreastatin-like sequence as C-terminal part. Its primary structure, as determined by peptide sequence analysis, is identical to the human CgA sequence, positions 210–301 (7). N-terminal to peptide hCgA-210–301, a Lys-Arg pair is located in human CgA that could serve as a proteolytic cleavage site, but it has to be proven whether this is being used in nontumor tissue *in vivo*. Proteolytic

FIG. 7. Immunocytochemical localization of PLI in human tissues and endocrine tumors. All sections were stained with antiserum pP-33–49-Glu13. (A) Human pancreatic islet; (B) human adrenal gland; (C) liver metastasis of the human carcinoid tumor from which human PLI was extracted and purified. ($\times 210$.)

processing of this peptide at three potential cleavage sites could result in a human pancreastatin peptide similar in size to the porcine analogue: cleavage at dibasic residues, at Arg-Lys (248–249) or Lys-Gly (249–250), or processing at a single arginine residue, at Arg-Ser (253–254), would produce pancreastatin-like peptides of 48, 52, or 53 residues, respectively. Only two putative processing sites at the N terminus of the pancreastatin sequence are conserved in all species: Lys-245 and Arg/Lys-248. It remains to be shown whether one of these residues serves as a uniform processing signal. The overall structural identity between the human, bovine, rat, and porcine pancreastatin region in CgA approaches 70%. Proteolytic processing at the C terminus of these peptides probably involves formation of intermediate glycine-extended molecules that may subsequently be converted to C-terminally amidated peptides. This process takes place at a Gly-Lys-Ser site (hCgA-302–304) that is conserved in human and bovine CgA. The same recognition sequence for C-terminal cleavage and amidation has been identified in the multicopy precursor of the *Aplysia* neuropeptide Phe-Met-Arg-Phe amide (24) and in guinea pig prepropancreatic polypeptide (25). Considering the fact that the C-terminal pancreastatin peptide pP-33–49 is slightly more potent in inhibiting insulin secretion (1) than intact pP-1–49, it is tempting to speculate that hCgA-210–301 may further be processed to small C-terminal pancreastatin-like fragments. However, except for hCgA-254–301, no such peptides have been detected in this tumor.

Despite prolonged digestion with trypsin, a substantial amount of peptide hCgA-254–300, 47 residues, could be isolated in intact form, although three potential tryptic cleavage sites after single Lys residues are present. It can be assumed that numerous negatively charged Glu residues in close proximity to basic amino acids may prevent effective interaction with trypsin and possibly with other trypsin-like processing enzymes.

Little is known about the *in vivo* proteolytic processing of CgA. In rat insulinoma cells cleavage of CgA leads to the formation of a 20-kDa N-terminal fragment, betagranin (26–28). A 62-kDa N-terminal fragment has been found in chromaffin granules of the adrenal medulla (29). Since six dibasic amino acid residues are clustered within the C-terminal domain of the bovine and human molecule, it has been proposed that proteolytic processing starts in this region (3, 29, 30), but peptides deriving from the mid- and C-terminal part of CgA have not been identified. Here, we demonstrate that proteolytic cleavage in a human carcinoid tumor may produce a large amidated peptide including pancreastatin, which is located C-terminally to the proposed processing site of betagranin within the CgA sequence. The identification of two more peptides derived from the C-terminal region of human CgA suggests that two pairs of basic amino acids may serve as proteolytic cleavage sites: Lys-Arg (338–339) and Arg-Arg (373–374). Trypsin-like cleavage after Arg-339 leads to the formation of peptide hCgA-P2, whereas hCgA-P3 probably results from cleavage after Arg-374 and partial degradation by a dipeptidyl aminopeptidase-like enzyme.

The isolation of these peptides raises several questions: Does CgA take part in the regulation of tissue-specific posttranslational processing of prohormones by acting as a substrate, regulator, or inhibitor for processing enzymes as has been claimed (31)? Is there any specific intracellular, paracrine, or endocrine regulatory function for CgA-derived peptides, as suggested by the inhibition of catecholamine secretion mediated by yet-undefined tryptic CgA peptides (32)? Do they simply represent degradation products with a certain pharmacological activity? Proteolytic processing of human CgA at some of the nine dibasic residues may lead to the formation of other yet-unidentified peptides. It remains to be shown whether these or other CgA fragments are biologically

active. The processing pathways for CgA in normal endocrine cells need to be defined by using sequence-specific antibodies. The structural characterization of these human CgA-derived peptides enables the clinical investigation of their role in various endocrine diseases.

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