Prosomatostatin-derived Antrin Is Present in Gastric D Cells and in Portal Blood

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Abstract

The three widely distributed peptides derived from proso-matostatin are the original neurohormone somatostatin-14, somatostatin-28, and somatostatin-28(1–12), which are all derived from the COOH terminus of the precursor. Recently a decapeptide derived from the NH₂ terminus of the prohormone has been identified in extracts of rat gastric antrum and named antrin. Data now show that in both rats and humans this new molecular form is concentrated in the D cell of the gastrointestinal mucosa together with somatostatin-28(1–12). The highest concentration of antrin immunofluorescent cells is located in the mucosa of the gastric antrum. Ultrastructural studies performed on pyloric D cells using the protein A-gold technique reveals that antrin is present in the same secretory granules as somatostatin-28(1–12) and detectable in one-third of all secretory granules. Acid extracts of rat hepatic portal plasma contain a peptide similar or identical to antrin, indicating that the antral decapeptide circulates in blood.

Introduction

The notion that peptide hormones are derived from precursor proteins by posttranslational modification is now well recognized (1). During the maturation process several peptides may be generated together with the hormone. These other peptides, often called cryptic peptides, are important for three reasons: (a) their presence provides information on the processing events, (b) some are better markers for a given peptidergic system than the hormone itself, and (c) among these peptides, a few could serve a biological role. After a new molecular form has been identified in a given tissue extract, several questions are raised, such as whether it may represent an artifact of extraction. If the peptide is not produced artifactually it is important to know the cell type from which it originates and whether it can be secreted by these cells.

Somatostatin is a bioactive peptide of 14 or 28 amino acids that generally acts as a paninhibitor in the neuroendocrine system. It is derived from the COOH-terminal end of a 10-kD precursor protein, prosomatostatin (2–5) (Fig. 1). We have recently isolated a peptide from rat stomach that corresponds to the first 10 amino acids at the NH₂ terminus of mammalian prosomatostatin (6). In the rat, the highest concentration of the decapeptide is present in the gastric antrum, hence the proposed name antrin. It is presently assumed that antrin is formed after cleavage at a monobasic site very similar to that leading to formation of somatostatin-28 and somatostatin-28(1–12). In the present work we provide the first immunocytochemical data on the cellular and ultrastructural localization of antrin and compare it with somatostatin-28(1–12), an excellent marker of the prosomatostatin system in tissue (7, 8). Evidence is also presented for the antral decapeptide circulating in hepatic portal blood in rats.

Methods

Antisera

To obtain specific antibodies that recognize only antrin, not proso-matostatin or any of the other prosomatostatin-derived peptides, one must direct the formation of gamma globulin toward the COOH terminus of antrin. This was achieved by immunizing rabbits with a mixture of synthetic antrin and methylated BSA or with the analogue [TYR³]antrin (3–10) coupled to BSA as described previously for several other peptides (9). The immuno plasma generated by the first method (Fig. 2) was selected for immunocytochemical studies (A09). An antibody population with higher affinity (A14) was obtained by the second method and it was selected for quantitation of antrin by RIA. A previously characterized antibody population (S320) against somato-statin-28(1–12) was used as a marker of the prosomatostatin system (7).

Immunocytochemistry

Light microscopy. Pieces of pancreas and gastrointestinal mucosa of normal adult Sprague-Dawley rats fixed by vascular perfusion with 1% glutaraldehyde were dehydrated and embedded in Epon. Consecutive semithin sections (1 μm) attached on glass slides were incubated by the indirect immunofluorescence method (10). Sections were treated to remove Epon (11) and then incubated for 2 h at room temperature with the primary antisera A09 (diluted 1:1,000) for antrin or S320 (diluted 1:4,000) for somatostatin-28(1–12) immunoreactivity. Sections were washed with PBS and then exposed to FITC-conjugated goat anti-rabbit IgG for 1 h. After washing and counterstaining with 0.03% Evans Blue the sections were examined with a fluorescence microscope (Axiophot; Carl Zeiss, Inc., Oberkochen, FRG). The procedure for the fixation and embedding of the human fetal tissue is detailed in reference 12. Sections were etched with 5% (vol/vol) H₂O₂ before immunofluorescence labeling. Control of specificity was carried out by applying anti-antrin serum previously absorbed with 10 μg/ml antrin, prosomatostatin(1–32), somatostatin-14, somatostatin-28, or somatostatin-28(1–12).

Electron microscopy. Thin sections attached on nickel grids were immunostained by the protein A-gold (pAg)[1] technique (13) with anti-

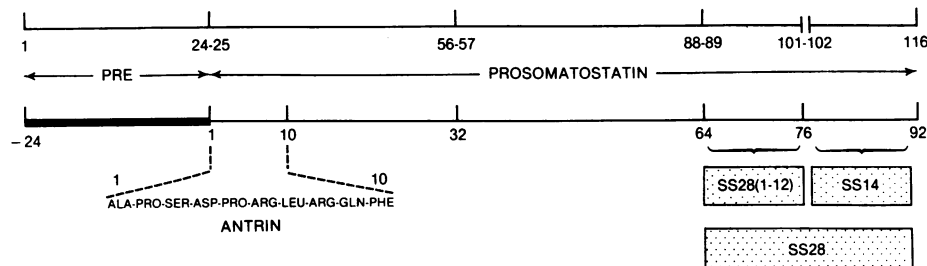1. *Abbreviations used in this paper:* pAg, protein A-gold.

Figure 1. Schematic representation of the 116–amino acid precursor of mammalian somatostatin. It contains a signal peptide or "pre" region of 24 amino acids preceding the 92 amino acid prosomatostatin of 10 kD. Antrin represents the first 10 amino acids at the amino terminus of prosomatostatin while somatostatin-14 represents the last 14 amino acids at the carboxyl terminus. Direct and indirect evidence indicates that cleavage occurs at Gly^{24}-Ala^{25}, Leu^{56}-Leu^{57}, Arg^{88}-Ser^{89}, and Arg^{101}-Lys^{102}. Antrin is probably generated through cleavage at Lys^{37}-Ser^{38} (not shown), a monobasic site also present in catfish as Arg^{37}-Ser^{38}.

antrin diluted 1:3,000 or anti-somatostatin diluted 1:4,000. Gold particles 10 nm in diameter were used. After immunolabeling sections were double stained with uranyl acetate and lead citrate and examined in an electron microscope (model 301; Philips Electron Optics, Eindhoven, The Netherlands).

The number of secretory granules expressing antrin immunoreactivity was quantified on photographic prints of 8–10 D cells (magnification of 34,000). 500–600 secretory granules were evaluated. Secretory granules showing at least four gold particles were counted as immunoreactive for antrin.

Gel permeation chromatography of hepatic portal plasma

Rat hepatic portal plasma was extracted with 2 M acetic acid, the extract was loaded onto octadecylsilylsilica cartridges and the retentate eluted with acetonitrile. The material eluted with acetonitrile was dried, dissolved in 5.2 M acetic acid, and loaded onto a Sephadex G-75 gel permeation chromotography column. Each column fraction was monitored for the presence of antrin using an RIA in which iodinated [TYR³]antrin(3-10) was used as the ligand.

Results

Immunofluorescence with A09 anti-antrin serum on sections of pancreas and gastrointestinal mucosa of rat revealed that antrin immunofluorescence was undetectable in pancreatic islets, seldom present in epithelial cells of oxyntic and duodenal mucosa, but clearly represented in endocrine cells of the gastric antrum (Fig. 3 *A*). All antrin immunofluorescent cells examined reacted also with S320 antibody. The staining for antrin was more sparse than that for somatostatin-28(1–12) which, when present, produced a diffuse cytoplasmic staining (compare Fig. 3, *A* and *B*). The same experiments performed in human fetal gastric tissue yielded similar results for both antrin and somatostatin-28(1–12) immunostaining (Fig. 3, *C* and *D*). Control of specificity showed that among the peptides used to adsorb the anti-antrin serum before the immunolabeling, only antrin abolished the staining.

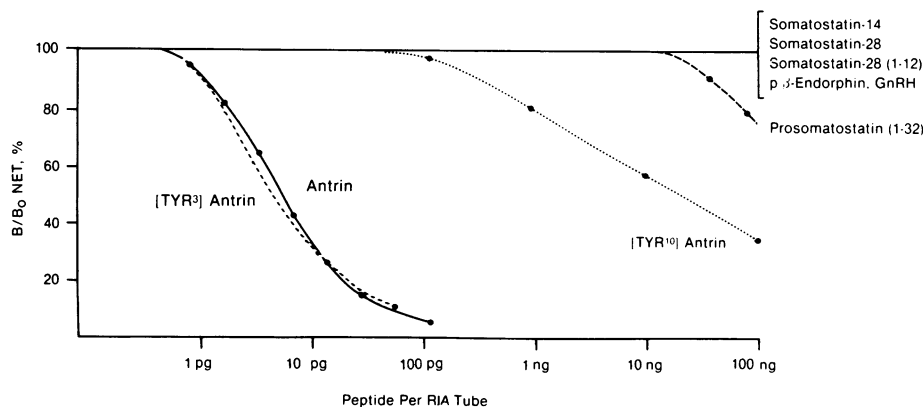At the ultrastructural level antrin immunoreactivity, local-

Figure 2. Characteristics of A09, an antibody population produced against antrin. A New Zealand White rabbit was immunized with an emulsion containing synthetic antrin (14), methylated BSA, and complete Freund adjuvant supplemented with 10 mg killed mycobacterium tuberculosis as previously described (9). Booster injections were done with the same emulsion but without bacteria. 10 d after the second boost the immune plasma was collected and used at a 1/100,000 dilution for RIA. The ligand was ^{125}I [TYR³]-antrin(3-10) which was obtained after iodination of 2.5 µg [TYR³]antrin(3-10) with 20 µg chloramine T during 38 s followed by addition of 75 µg sodium metabisulfite and 1 mg human serum albumin. The label was purified on carboxymethyl cellulose (CM 52; Whatman Laboratory Products, Inc., Clifton, NJ) using 0.04 M ammonium acetate, pH 4.6, as the elution buffer. Net binding of label to antibody was 31% after 44 h incubation at 4°C in RIA buffer (9). The specificity studies represented here show that cross-reactivity with preproSS(25–33)-TYR (i.e., [TYR¹⁰]antrin) and prosomatostatin(1–32) is 0.03% and 0.003%, respectively, indicating that A09 recognizes the COOH-terminal region of antrin. This was confirmed by incubating ^{125}I-[TYR¹⁰]antrin with A09 diluted at 1/10,000. No binding was observed (data not shown).
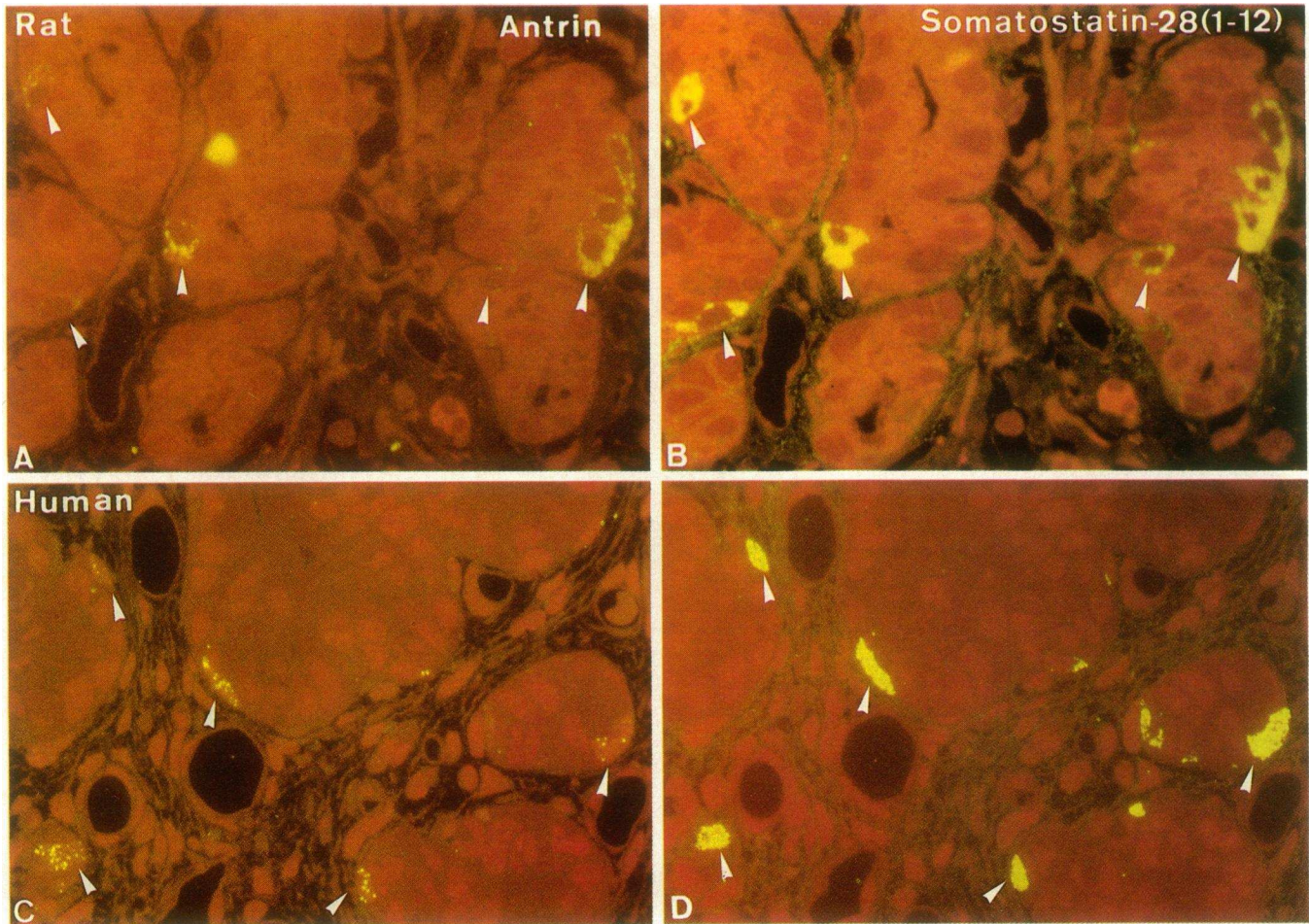
Figure 3. Localization of antrin and somatostatin-28(1–12) immunoreactivity by immunofluorescence on consecutive semithin sections of antral mucosa from adult rat (*A, B*) and 17-wk human fetus (*C, D*). The same D cells (*arrowheads*) show antrin and somatostatin-28(1–12) immunoreactivity. However, the pattern of antrin immunolabeling of individual cells appears rather sparse compared with the very bright and compact immunofluorescence of somatostatin-28(1–12). *A, B:* ×800; *C, D:* ×630.

ized by pAg, was observed over the secretory granules. Labeled granules frequently coexisted with granules devoid of immunolabeling (Fig. 4, *A* and *C*). Quantitative evaluation of antrin-positive granules showed that they represented 33% of the population of secretory granules in rat D cells and 15% in human fetal D cells.

Comparing serial sections, one incubated with anti-antrin and the other with anti-somatostatin-28(1–12), it was found that the secretory granules containing antrin also contained somatostatin-28(1–12) (Fig. 4, *A–D*). By contrast, many somatostatin-positive granules do not contain antrin. Inasmuch as the antiserum recognizes the COOH terminus of prosomatostatin 1–10 (antrin) it appears that the processing of prosomatostatin to antrin occurs in only a subpopulation of the granules that process prosomatostatin to prosomatostatin-28(1–12). Antrin could be a late cleavage product, occurring only in "old" granules, and never be secreted. When extract of rat hepatic portal plasma was subjected to gel permeation chromatography, it was found that a single peak of immunoreactivity eluted in a zone compatible with a peptide the size of antrin (~ 1,200 mol wt) (Fig. 5).

Discussion

The findings obtained with a specific antibody population directed toward antrin indicate (*a*) that the decapeptide is present in secretory granules that also contain somatostatin-28(1–12), and (*b*) that it is detectable in only a fraction of somatostatin-containing granules in a subpopulation of somatostatin-producing cells. This suggests differential precursor processing or product stability within the granules, and antrin could thus be a marker for the presence of specific cleavage enzymes.

Antrin cannot be detected by immunohistochemistry in gastric nerves, nor can it be detected in the D cells of the pancreas. This restricted tissue distribution, namely that the endocrine cells of the stomach are the predominant site of antrin accumulation, suggests that the measurement of antrin in blood may represent a means by which to evaluate specifically the response of the gastric D cell during digestion. Together with the demonstration of this peptide in the portal circulation, it also suggests that antrin acts as a hormone during digestion. The high degree of amino acid sequence conser-
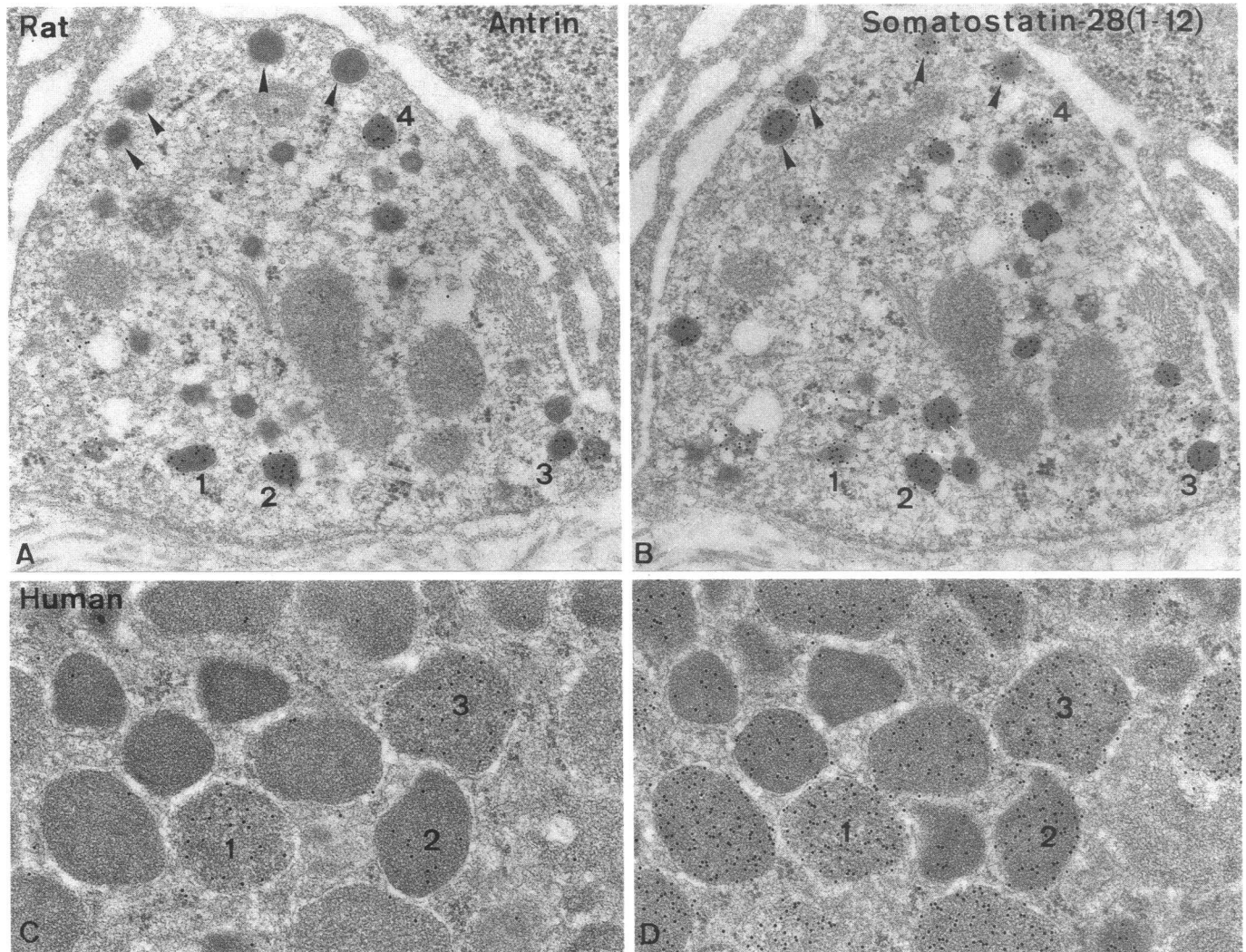
Figure 4. Localization of antrin and somatostatin-28(1-12) at the ultrastructural level on consecutive thin sections of antral D cells of rat (*A, B*) and human fetus (*C, D*) processed by the pAg method. In electron microscopy antrin was revealed over a population (33% in rat, 15% in human) of secretory granules while somatostatin-28(1-12) was present on virtually all the secretory granules. Serially cut secretory granules containing antrin and somatostatin-28(1-12) immunoreactivity are numbered. Arrowheads point to secretory granules with somatostatin-28(1-12) but not antrin immunolabeling. *A, B:* ×34,000; *C, D:* ×40,000.
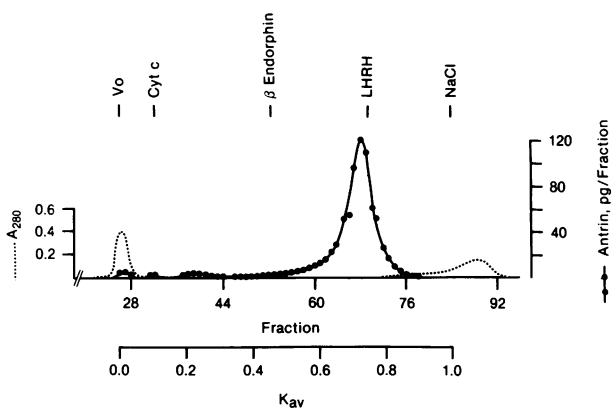

Figure 5. Sephadex G-75 gel permeation chromatography of an acid extract of 78 ml rat portal plasma. Hepatic portal blood was collected in cold heparinized syringes from adult Sprague-Dawley rats fed ad lib. After centrifugation at 12,800 g for 2 min at 4°C the plasma was added to 2 M acetic acid (1 vol. for 1 vol.), vortexed, frozen in liquid nitrogen, and kept at −20°C until purification on Sep-Paks (Waters Associates, Milford, MA). The material eluted from octadecylsilylsilica cartridges with 80% acetonitrile in 0.1% trifluoroacetic acid was lyophilized. The dry powder was dissolved in 5.2 M acetic acid and loaded onto a Sephadex G-75 column (2.6 × 107 cm). Antrin radioimmunoactivity was measured with antibody A14 in fractions 23–92. A major peak of immunoreactive material was detected at 0.7 K_{av} $[(V_x - V_0)/(V_{salt} - V_0)]$, which is compatible with a peptide of 1,200–1,300 mol wt. The column was previously calibrated with cytochrome *c*, 12,384 mol wt, human β-endorphin, 3,466 mol wt, luteinizing-hormone releasing hormone (LHRH), 1,182 mol wt, and NaCl, 58.5 mol wt. The peak seen in the void volume represents crystalline BSA (Pentex; Miles Laboratories, Inc., Elkhart, IN) 67,000 mol wt, used for coating the column. Flow rate was 21 ml/h and fraction size was 7 ml.

vation from catfish to man in the region of prosomatostatin corresponding to antrin argues in favor of a biological role for that molecule (5).

Acknowledgments

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