Secretogranins I and II: Two Tyrosine-Sulfated Secretory Proteins Common to a Variety of Cells Secreting Peptides by the Regulated Pathway

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ABSTRACT We report on the biochemical and immunological properties as well as on the cellular and subcellular distribution of two proteins, called secretogranins I and II. These proteins specifically occur in a wide variety of endocrine and neuronal cells that package and sort regulatory peptides into secretory granules. Both secretogranins take the same intracellular route as the peptides and are also sorted into secretory granules. Secretogranins I and II are biochemically and immunologically distinct proteins and differ from chromogranin A. Yet, these three proteins are similar to each other in many respects and therefore constitute one class of proteins. A remarkable feature of this protein class is a very acidic pI, brought about by a high content of acidic amino acids as well as by phosphorylation on serine and sulfation on tyrosine and O-linked carbohydrate. As a result, this class of proteins has a high net negative charge even at the acidic pH of the *trans* Golgi cisternae. We discuss the possibility that this property of the proteins may point to a role in the packaging of regulatory peptides into secretory granules.

The intracellular process by which peptide hormones and neuropeptides are secreted has been intensively studied, and many aspects of this process have been characterized (for reviews see references 6, 14, and 43). However, some important questions have remained unanswered, such as how peptides that are secreted by the regulated pathway (16, 32) are packaged and sorted into secretory granules. It can be expected that the identification of specific features of the secretory process that are common to endocrine and neuronal cells, irrespective of the specific peptide secreted, will help to elucidate the unknown mechanisms of packaging and sorting of regulatory peptides.

We have been interested (*a*) in posttranslational modifications that specifically take place at the intracellular sites where packaging and sorting of peptides are known to occur, and (*b*) in the possible existence of target proteins for these modifications that are common to different peptide-secreting endocrine and neuronal cells. Previous work has shown that the covalent modification of secretory proteins by sulfation of tyrosine (18, 20) apparently occurs in the Golgi complex at the level of the *trans* cisternae (28; Baeuerle, P. A., and W. B. Huttner, unpublished observations). The search for the major tyrosine-sulfated proteins in a model neurosecretory cell line, the rat pheochromocytoma cell PC12, led to the identification of two previously uncharacterized secretory proteins, one consisting of a pair of homologous polypeptides of 113/105 kD, and the other one consisting of a pair of homologous polypeptides of 86/84 kD (27).

Other studies, investigating sulfated macromolecules in secretory granules, led to the identification of a previously unknown secretory protein of the bovine anterior pituitary (36, 48). An immunologically related protein appeared to be present in the adrenal medulla (37), the tissue from which PC12 cells are derived. We have recently shown that the anterior pituitary protein also consists of a pair of homologous polypeptides of 86/84 kD that contain the sulfate bound to tyrosine residues. These results suggested that the 86/84-kD

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protein of PC12 cells may be identical to the 86/84-kD protein of the anterior pituitary. Moreover, since this protein was found in secretory granules of different endocrine cells of the anterior pituitary (38), we considered the possibility that this protein may not only occur in the anterior pituitary but may be common to a wide variety of endocrine and neuronal cells that secrete peptides by the regulated pathway.

Recent reports have shown that chromogranin A, a 75-kD secretory protein first found in chromaffin granules (for reviews see references 44 and 45), occurs in many but not all endocrine and neuronal cells (7, 8, 33, 41). Considering these reports and our previous results, it occurred to us that (*a*) the 86/84-kD protein, the 113/105-kD protein, and chromogranin A may be members of one class of proteins, (*b*) the distribution of this protein class may be more widespread than that of any one member, and (*c*) the individual members may share certain properties, the identification of which may provide essential clues as to their function.

We now report that the 86/84-kD protein and the 113/105-kD protein indeed occur in secretory granules of a wide variety of peptide-secreting endocrine and neuronal cells, and that their cellular distribution is partly overlapping and partly complementary. In view of their cellular and subcellular localization, we have named the 113/105-kD protein secretogranin I and the 86/84-kD protein secretogranin II. The results demonstrate that secretogranin I, secretogranin II, and chromogranin A are distinct proteins but are sufficiently similar in many respects to be regarded as one protein class.

MATERIALS AND METHODS

Tissue Preparation and Subcellular Fractionation: Bovine and rat tissues were dissected, frozen in liquid nitrogen, crushed on dry ice to a fine powder, homogenized 1:10 (wt/vol) in sample buffer (25, 27), and immediately boiled. Chromaffin granules were isolated from bovine adrenal medulla as described (3). The isolated granules were lysed by resuspension in 20 mM Tris-HCl, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, followed by two cycles of freezing and thawing. The suspension was centrifuged for 1 h at 140,000 *g*, and the soluble fraction was collected. In some experiments, the heat-stable protein fraction was prepared from the soluble fraction of chromaffin granules as described below.

Purification of Secretogranins I and II and Chromogranin A: Tissues were homogenized 1:10 (wt/vol) in 20 mM Tris-HCl, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and the homogenates were centrifuged at 140,000 *g* for 1 h. To the soluble fractions obtained, NaCl was added to a final concentration of 150 mM, followed by boiling for 5 min and centrifugation as above. The resulting supernatant is referred to as heat-stable protein fraction. For polyacrylamide gel electrophoresis, the heat-stable proteins, highly enriched in secretogranins I and II and chromogranin A, were concentrated by acetone precipitation and resuspended either in sample buffer or in lysis buffer (35).

For raising antisera, bovine secretogranin II was purified from the heat-stable protein fraction of anterior pituitary by DEAE-cellulose chromatography (37) and preparative two-dimensional PAGE. Bovine secretogranin I and chromogranin A were purified from the soluble fraction of chromaffin granules by preparative two-dimensional PAGE. Rat secretogranins I and II were purified from PC12 cells as described in detail elsewhere (Hille, A., and W. B. Huttner, unpublished observations). Briefly, the purification procedure involved hypotonic lysis of PC12 cells, heat treatment of the soluble protein fraction, DEAE-cellulose chromatography, and preparative two-dimensional PAGE.

For the experiment shown in Fig. 8, secretogranin I and II were enriched from various rat tissues as follows. Tissues were homogenized 1:10 (wt/vol) in 50 mM Tris-HCl, pH 7.5, 1% (wt/vol) SDS, 5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, boiled for 5 min, and centrifuged at 10,000 rpm for 30 min. Aliquots of the supernatants (2–4 mg total protein) were diluted 1:10 in 50 mM Tris-HCl, pH 7.5, 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) deoxycholate, and 5 mM EDTA, and incubated with 25 μl of anti-rat secretogranin I antiserum plus 25 μl of anti-rat secretogranin II antiserum (see below). The immune complexes were absorbed to *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring Corp., LaJolla, CA) as described (22) and extensively washed. Secretogranins I and II were separated from the *Staphylococcus aureus* cells and the immunoglobulins by boiling in the presence of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3% (vol/vol) 2-mercaptoethanol, and 10 mM EDTA, followed by centrifugation in a microfuge (Eppendorf, Hamburg, FRG). The heat-stable proteins present in the resulting supernatant were precipitated with acetone, solubilized in sample buffer, and subjected to SDS PAGE followed by immunoblotting (see below).

Antisera: After preparative two-dimensional PAGE, gels were either stained by the KCl method (17) or fixed, stained with Coomassie Blue, and destained (27). Pieces containing either rat or bovine secretogranin I, secretogranin II, or chromogranin A were cut from the gels, homogenized in a small volume of phosphate-buffered saline (PBS), and used for immunization of rabbits. Gel homogenates were emulsified 1:1 with complete Freund's adjuvant for the first injection and with incomplete Freund's adjuvant for the booster injections. 50–100 μg of purified protein were used per injection.

Cell Culture, Labeling, and Release of Secretogranins: PC12 cells were grown as previously described (27). The primary culture of bovine adrenal medullary cells was performed as described (1). [^{35}S]Sulfate- and [^{32}P]phosphate-labeling of cell cultures was performed for 18–24 h as described (27). In the case of PC12 cells, the medium was supplemented with 10% dialyzed horse serum and 5% dialyzed fetal calf serum; in the case of adrenal medullary cells, the medium was supplemented with 1.5% dialyzed horse serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate (P0781, Sigma Chemie, Taufkirchen, FRG). Dialysis of the sera was against PBS for sulfate labeling and against HEPES-buffered saline for phosphate labeling. In some experiments, [^{35}S]sulfate labeling of adrenal medullary cells was performed in the presence of tunicamycin (Boehringer Mannheim Biochemicals, Mannheim, FRG) (1.5 μg/ml).

Uncoated 35-mm dishes containing 1×10^6 [^{35}S]sulfate-labeled PC12 cells were washed with Dulbecco's modified Eagle's medium (Gibco Europe, Karlsruhe, FRG) and incubated for 15 min in 0.5 ml of release medium (10 mM HEPES, pH 7.2, 2.2 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM glucose, and either 127 mM NaCl/5 mM KCl, 77 mM NaCl/55 mM KCl, or 67 mM NaCl/55 mM KCl/10 mM $MgCl_2$). The release medium was collected and centrifuged at room temperature for 5 min at 100 *g*; the supernatant was further cleared by centrifugation at 4°C for 30 min at 100,000 *g*. The cells attached to the dish were lysed and collected in 0.5 ml of 50 mM Tris-HCl, pH 7.5, 0.5% (wt/vol) Triton X-100, 150 mM NaCl, 5 mM EDTA, and 0.02% NaN_3. The suspension was rapidly frozen, thawed, sonicated for 10 s, centrifuged at 4°C for 30 min at 43,000 *g*, and the supernatant was collected. Aliquots of this supernatant (cell extract) (10 μl, 0.23 μg/ml of protein) and of the release medium (200 μl) were incubated with 15 μl of a mixture of anti-rat secretogranin I and anti-rat secretogranin II antisera in the presence of 0.5% (wt/vol) Triton X-100 and 150 mM NaCl. The amount of mixed antisera was sufficient to bind all of secretogranins I and II present in the samples. Immune complexes were collected by binding to *Staphylococcus aureus* cells (22), solubilized in sample buffer, and subjected to SDS PAGE. [^{35}S]Sulfate-labeled secretogranins I and II were cut from the gel and quantitated by liquid scintillation counting.

In Vitro Translation of Secretogranins I and II: Free and membrane-bound polysomes from PC12 were prepared as described (31), with minor modifications. The composition of buffer A was changed to 0.3 M KCl, 5 mM $MgCl_2$, 0.25 M sucrose, and 50 mM Tris-HCl, pH 7.4 and that of buffer B to 0.3 M NaCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4. 5×10^8 PC12 cells were washed with Dulbecco's modified Eagle's medium and pelleted by centrifugation. The cell pellet was frozen in liquid nitrogen, homogenized in 15 ml of buffer A, centrifuged for 10 min at 12,000 *g*, and the resulting supernatant was subjected to discontinuous sucrose gradient centrifugation. The pellet containing free polysomes was resuspended in 50 μl of buffer B (A_{260}/A_{280} = 1.810). The material at the 1.0–2.0 M sucrose interface that contained bound polysomes was collected, incubated in the presence of 1% (wt/vol) Triton X-100 and 1% (wt/vol) deoxycholate for 1 h at 4°C, layered over 20 ml of 2.0 M sucrose in buffer A, and centrifuged for 16 h at 95,000 *g*. The pellet containing bound polysomes was resuspended in 50 μl of buffer B (A_{260}/A_{280} = 1.634). In vitro translation was performed as described (31). One A_{260} unit of polysomes and 100 μCi of [^{35}S]methionine (800 Ci/mmol) (New England Nuclear, Du Pont, Dreieich, FRG) were used per reaction. After incubation for 90 min at 25°C, reactions were terminated by freezing. Samples were lyophilized, solubilized in lysis buffer, and aliquots were analyzed by two-dimensional PAGE followed by fluorography.

SDS PAGE, Two-dimensional PAGE, and Immunoblotting: SDS PAGE and two-dimensional PAGE were performed according to Laemmli (25) and O'Farrell (35), respectively. For satisfactory resolution of secretogranins I and II and chromogranin A, a combination of ampholytes (LKB, Gräfelfing, FRG) was used for isoelectric focusing as described previously (38). Gels

were fixed, stained, destained, and fluorographed as described (27). Immunoblotting after SDS PAGE or two-dimensional PAGE was performed as described (5), with the modifications described elsewhere (30). All antisera were used at a 1:100 dilution.

Tryptic Fingerprinting: Proteins were excised from fixed, stained, destained, and dried two-dimensional polyacrylamide gels and individually subjected to radioiodination and tryptic fingerprinting as described (11), with minor modifications (38).

Tyrosine Sulfate and Phosphoamino Acid Analysis: [^{35}S]-Sulfate-labeled and [^{32}P]phosphate-labeled proteins were excised from fixed, stained, destained, and dried two-dimensional polyacrylamide gels and subjected to tyrosine sulfate analysis (21) and phosphoamino acid analysis (19), respectively.

Amino Acid Analysis: Secretogranin II was purified from bovine anterior pituitary, eluted from preparative SDS polyacrylamide gels, and precipitated by acetone as described previously (37). Material eluted and precipitated from an equal quantity of a blank gel was analyzed as a control. The precipitates were hydrolyzed for 18 h in 6 N HCl at 110°C. The hydrolyzates were dried, re-dissolved in 0.2 M citrate buffer, pH 2.2, and amino acids were then analyzed in a Multichrom M Beckman automatic analyzer (Beckman Instruments, Inc., Palo Alto, CA) according to the method of Spackman et al. (42). Amino acid values obtained for secretogranin II were corrected for those obtained for the gel blank.

Immunocytochemistry: Anterior pituitaries, dissected from female rats, were immediately fixed in ice-cold formaldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in Epon (38). In other experiments, male and female rats were anesthetized and transcardially perfused with saline, followed by perfusion with 120 mM phosphate buffer, pH 7.4, 4% (wt/vol) formaldehyde, and 2.5% (wt/vol) polyvinyl pyrrolidon. Tissues were then dissected, incubated for 4 h in the same ice-cold fixative, and subsequently either passed through a series of increasing sucrose solutions and used to prepare frozen sections (29) or dehydrated and embedded in Epon. Indirect immunofluorescence on plastic sections, after removal of Epon, and on frozen sections was performed as described (38), using rhodamine-conjugated goat IgG directed against rabbit IgG (Cappel Laboratories, Cochranville, PA) in the second antibody step. Immunolabeling of frozen sections of the brain, using horseradish peroxidase–conjugated goat F(ab)$_2$ anti-rabbit IgG (Institute Pasteur, Paris, France) in the second antibody step, was performed as described (9).

RESULTS

Presence of Secretogranin I, Secretogranin II, and Chromogranin A in Anterior Pituitary and Adrenal Medulla

Secretogranins I and II were previously shown to remain in solution when the soluble fraction of a PC12 cell lysate was boiled (26). In the present study, we have exploited this property to obtain fractions greatly enriched in secretogranin I, secretogranin II, and chromogranin A. To demonstrate the presence of all three proteins in both anterior pituitary and adrenal medulla, the heat-stable protein fractions of bovine anterior pituitary, bovine chromaffin granules, and as reference, [^{35}S]sulfate-labeled PC12 cells were analyzed by two-dimensional PAGE (Fig. 1). In mixing experiments, secretogranin II (arrows) of PC12 cells (Fig. 1 *B*) co-migrated with secretogranin II of the bovine anterior pituitary (Fig. 1 *A*). Secretogranin II was the major heat-stable protein of this tissue. Secretogranin II was also present, though in much smaller amounts, among the heat-stable proteins of bovine chromaffin granules (Fig. 1, *C–E*; compare also Fig. 2 *A*). Like secretogranin II, secretogranin I (arrowheads) was observed not only in PC12 cells (Fig. 1, *B* and *D*) but also (as a single 105-kD polypeptide) in the bovine anterior pituitary (Fig. 1 *A*) and in bovine chromaffin granules (Fig. 1 *C*). Chromogranin A (open arrows), in turn, was seen not only in bovine chromaffin granules (Fig. 1 *C*), but also, though in smaller amounts, among the heat-stable proteins of the bovine anterior pituitary (Fig. 1, *A* and *E*).

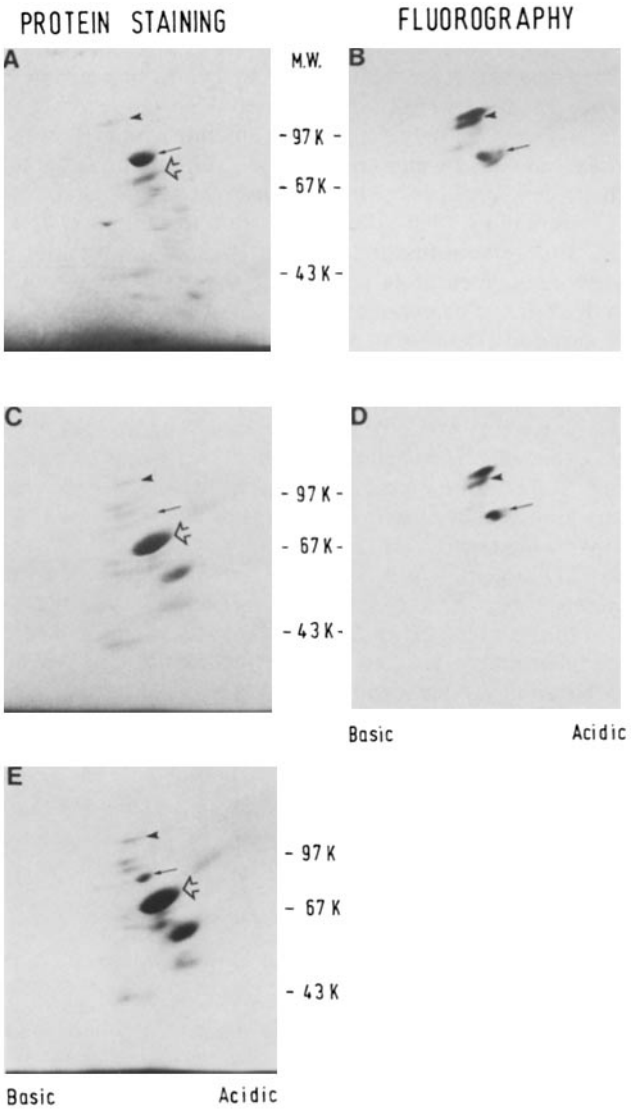

FIGURE 1 Presence of secretogranin I, secretogranin II, and chromagranin A in anterior pituitary and adrenal medulla. The heat-stable protein fractions of bovine anterior pituitary of chromaffin granules from bovine adrenal medulla, and of [^{35}S]sulfate-labeled PC12 cells were mixed. (*A* and *B*) Anterior pituitary (70 μg protein) plus PC12 cells (5 μg protein): (*C* and *D*) adrenal medulla (50 μg protein) plus PC12 cells (5 μg protein): (*E*) anterior pituitary (30 μg protein) plus adrenal medulla (50 μg protein). Samples were analyzed by two-dimensional PAGE, followed by Coomassie Blue staining (*A*, *C* and *E*) and fluorography (*B* and *D*). Arrowheads, 105-kD component of secretogranin I; arrows, secretogranin II; open arrows, chromagranin A. Note that the bovine secretogranin I apparently lacks the 113-kD component seen in the rat secretogranin I. The positions of molecular weight standards are indicated.

Secretogranin I, Secretogranin II, and Chromogranin A Are Three Biochemically Distinct Proteins

Secretogranin I, secretogranin II, and chromogranin A of the bovine adrenal medulla and of the bovine anterior pituitary were separated by two-dimensional PAGE (Fig. 2, *A* and *B*, respectively), individually radioiodinated, and analyzed by tryptic fingerprinting (Fig. 2, square panels). For each of the three proteins, the fingerprint of the adrenal medulla form was virtually identical to that of the anterior pituitary form.

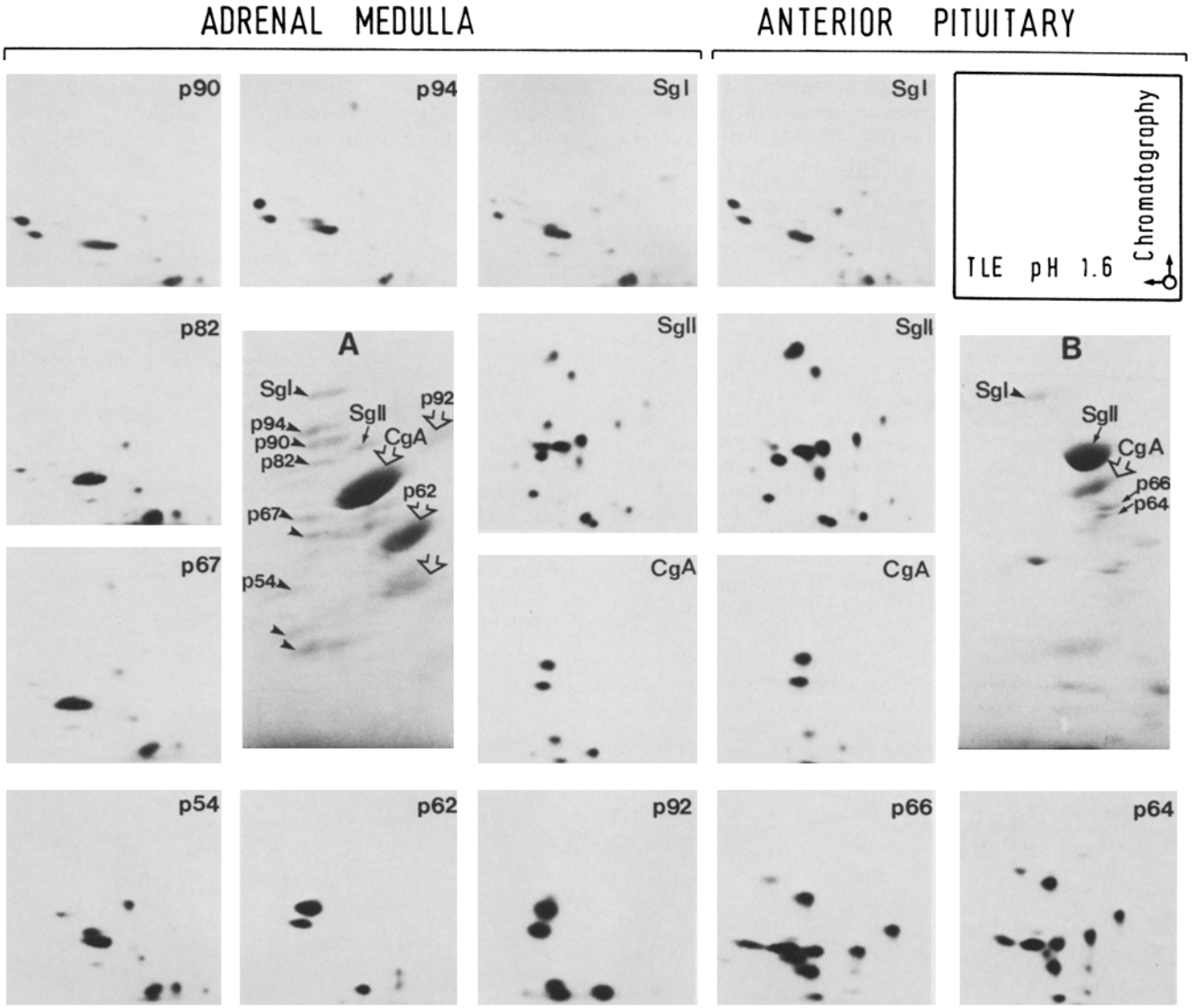

FIGURE 2 Secretogranin I, secretogranin II, and chromogranin A are three distinct proteins. The heat-stable protein fractions of chromaffin granules from bovine adrenal medulla (*A*) and of bovine anterior pituitary (*B*) were separated by two-dimensional PAGE followed by Coomassie Blue staining as shown. Individual proteins, indicated in panels *A* and *B*, were radioiodinated and analyzed by tryptic fingerprinting followed by fluorography (square panels). Arrowheads point to secretogranin I (*SgI*) and its related polypeptides; the three unlettered arrowheads in *A* indicate p63, p45, and p43, the fingerprints of which are not shown. Arrows point to secretogranin II (*SgII*) and its related polypeptides. Open arrows point to chromogranin A (*CgA*) and its related polypeptides; the unlettered open arrow in *A* indicates p51, the fingerprint of which is not shown. The two-dimensional separation system used for the radiolabeled tryptic peptides is shown in the diagram in the top right corner. *TLE*, thin-layer electrophoresis; cathode on the left.

However, the fingerprints of the three proteins were clearly different from each other. In fact, mixing experiments (not shown) indicated that very few, if any, of the radiolabeled peptides of the three proteins co-migrated exactly.

Fingerprint analysis was also used to investigate the relationship of the various other heat-stable proteins to secretogranins I and II and chromogranin A (Fig. 2). This revealed three distinct sets of proteins: (*a*) p94, p90, p82, p67, p63 (not shown), p54, p45 (not shown), and p43 (not shown) of adrenal medulla were related to secretogranin I; (*b*) p66 and p64 of the anterior pituitary were related to secretogranin II; (*c*) p92, p62, and p51 (not shown) of chromaffin granules were related to chromogranin A. The results on p62 and p51 confirm those of O'Connor and Frigon (34) who refer to these two proteins as chromogranins B and C, respectively. P92 appears to be a highly sulfated form of chromogranin A (see Figs. 4 and 6).

Secretogranin I, Secretogranin II, and Chromogranin A Are Three Immunologically Distinct Proteins

Antisera were raised in rabbits against rat secretogranins I and II purified from PC12 cells, bovine secretogranin II purified from anterior pituitary, and bovine secretogranin I and chromogranin A purified from adrenal medulla. To ensure purity of the antigens, these proteins were excised from two-dimensional polyacrylamide gels. One-dimensional immunoblotting of total PC12 cell protein (Fig. 3, lanes *1* and

2) and of total bovine anterior pituitary protein (Fig. 3, lane 3) showed that the various antisera were specific and recognized either secretogranin I, secretogranin II, or chromogranin A. The use of these antisera in two-dimensional immunoblots indicated the presence of three distinct sets of proteins in bovine and rat adrenal medulla (Fig. 4) and anterior pituitary (data not shown). These data supported the fingerprint results (Fig. 2) on the relationship of the various polypeptides to either secretogranin I, secretogranin II, or chromogranin A. It should be noted that the apparent molecular weight of rat chromogranin A was higher than that of bovine chromogranin A and was almost identical to that of rat secretogranin II (Fig. 4, *E* and *H*). The cross-reactivity of antisera against rat antigens with the respective bovine antigens was variable, and vice-versa.

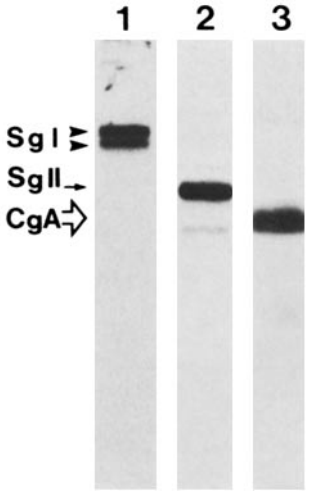

FIGURE 3 Specificity of antisera raised against secretogranin I, secretogranin II, and chromogranin A. Aliquots (100 μg protein) of the total homogenate of rat PC12 cells (lanes *1* and *2*) and of bovine anterior pituitary (lane *3*) were used for one-dimensional immunoblot analysis. Lane *1*, anti-rat PC12 cell secretogranin I antiserum; lane *2*, anti-rat PC12 cell secretogranin II antiserum; lane *3*, anti-bovine adrenal medulla chromogranin A antiserum. The positions of secretogranin I (*SgI*), secretogranin II (*SgII*), and chromogranin A (*CgA*) are indicated. Note that the faint band below secretogranin II in lane *2* is not chromogranin A but p66, one of the degradation products of secretogranin II (see Fig. 4).

In Vitro Translation of Secretogranins I and II

Free and membrane-bound polysomes of PC12 cells were isolated and, after solubilization of membranes, separately used in in vitro translation experiments (Fig. 5). This indicated that the 113- and 105-kD components of rat secretogranin I and the 86- and 84-kD components of rat secretogranin II were synthesized exclusively by polysomes derived from the membrane-bound population (Fig. 5 *B*). Since the mobilities of the in vitro translation products were not identical to those of the [^{35}S]sulfate-labeled proteins produced by PC12 cells, the identity of the translation products as secretogranins I and II was confirmed by immunoprecipitation using specific anti-rat secretogranin I and anti-rat secretogranin II antisera (Benedum, U. M., and W. B. Huttner, unpublished data).

Secretogranin I, Secretogranin II, and Chromogranin A Are Phosphorylated and Sulfated

Like chromogranin A (7), secretogranins I and II appeared

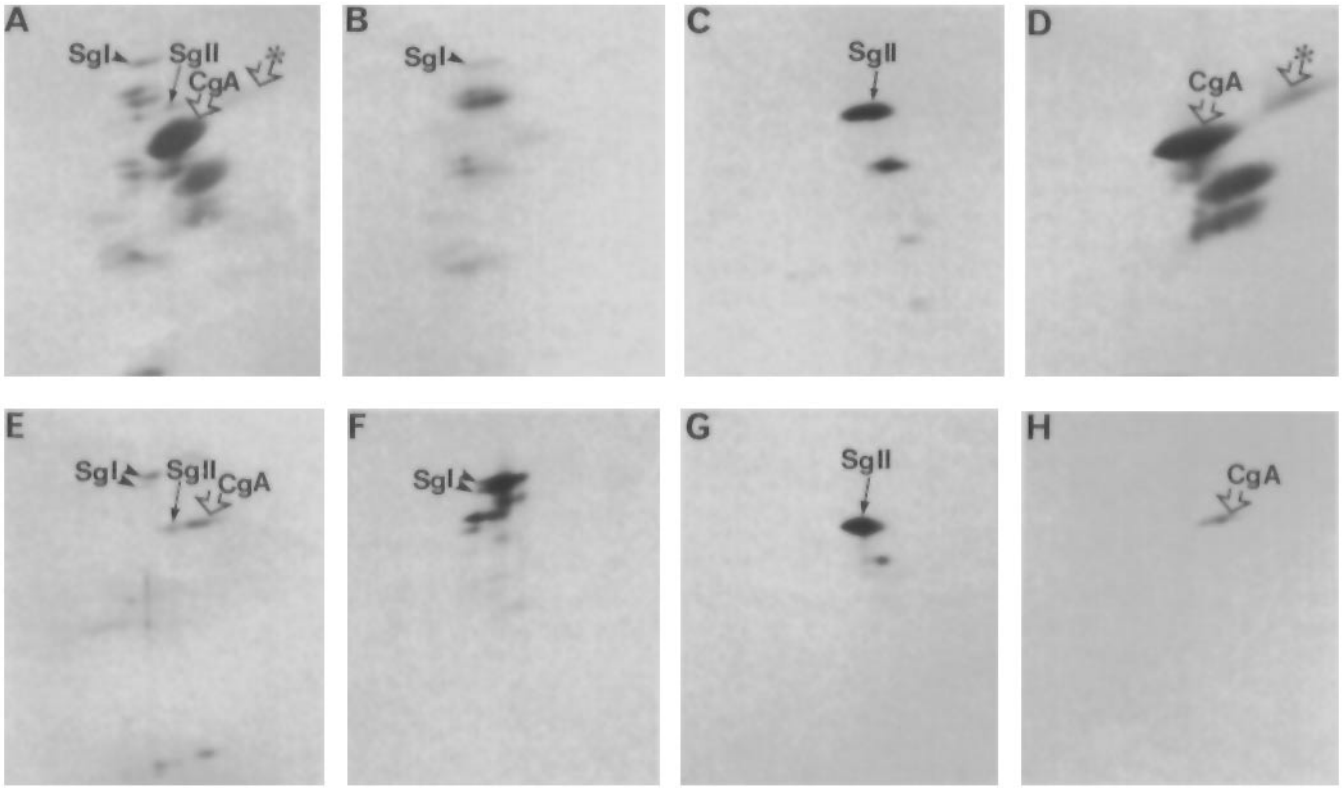

FIGURE 4 Immunological identification of bovine and rat secretogranin I-, secretogranin II-, and chromogranin A-derived proteins. Aliquots of either the soluble content of chromaffin granules from bovine adrenal medulla (*A–D*) or the heat-stable protein fraction of the rat adrenal gland (*E–H*) were used for two-dimensional immunoblot analysis. (*A* and *E*) Amido black staining of nitrocellulose filters; (*B–D* and *F–H*) fluorograms of nitrocellulose filters. (*B* and *F*) Anti-bovine (*B*) and anti-rat (*F*) secretogranin I antiserum; (C and G) anti-bovine (C) and anti-rat (*G*) secretogranin II antiserum; (*D* and *H*) anti-bovine chromogranin A antiserum. Arrowheads, secretogranin I (*SgI*); arrows, secretogranin II (*SgII*); open arrows, chromogranin A (*CgA*); open arrows with asterisk, highly sulfated form of chromogranin A (see Figs. 2 and 5).

to be rich in acidic amino acid residues since they yielded high proportions of glutamic acid and aspartic acid after acid hydrolysis (Table I and unpublished results). As a result, the translation products of the secretogranin I-secretogranin II-, and chromogranin A-mRNAs are acidic polypeptides (Fig. 5; reference 12). We have investigated the posttranslational modification of these proteins by phosphorylation and sulfation, which would further decrease the isoelectric points of these proteins. For this purpose, primary cultures of bovine adrenal medullary cells were labeled with [^{32}P]phosphate and [^{35}S]-sulfate. Analysis of proteins by two-dimensional PAGE indicated that secretogranins I and II and chromogranin A were phosphorylated (Fig. 6*A*), the phosphate in all three proteins being predominantly bound to serine residues and to a small extent to threonine, but not to tyrosine, residues (data not shown). The phosphorylation of chromogranin A confirms previous results by others (4, 40).

The chromogranin A spot also contained a small, but significant amount of radioactive sulfate (Fig. 6*B*). Interestingly, p92 (indicated in Fig. 6*B* by the open arrow with asterisk), which yielded the same fingerprint as chromogranin A (Fig. 2) and was specifically recognized by anti-chromogranin A antibodies (Fig. 4), contained a large amount of radioactive sulfate, suggesting that it was a highly sulfated form of chromogranin A. Secretogranins I and II were clearly sulfated (Fig. 6*B*). The radioactive sulfate incorporation into secretogranins I and II and chromogranin A was not reduced by labeling cells in the presence of tunicamycin, an inhibitor of N-glycosylation (data not shown). The sulfate found in chromogranin A and in the highly sulfated form of chromogranin A was not recovered to any significant extent as tyrosine sulfate (Table II). Together with the tunicamycin result, this indicated that the sulfate was bound to O-linked carbohydrate. A large portion, but not all, of the sulfate

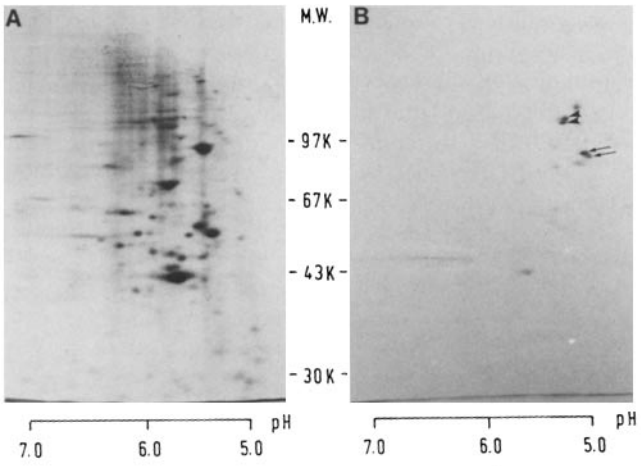

FIGURE 5 In vitro translation of secretogranin I and secretogranin II of PC12 cells. Free and bound polysomes were isolated from PC12 cells and incubated in the presence of [^{35}S]methionine. Proteins translated from free polysomes (*A*) and from bound polysomes (*B*) were analyzed by two-dimensional PAGE, followed by fluorography. Arrowheads, precursors of secretogranin I; arrows, precursors of secretogranin II.

TABLE I. *Amino Acid Composition of Bovine Secretogranin II: Comparison with That of Chromogranin A*

	Residues/100 residues	
	Secretogranin II*	Chromogranin A‡
Asx	13.71 ± 0.95	8.0
Glx	22.01 ± 1.62	22.5
Ser	6.42 ± 0.45	7.7
Thr	3.72 ± 0.21	2.6
Cys	n.a.	0.2
Met	0.40 ± 0.09	1.4
Pro	5.90 ± 1.08	9.2
Gly	6.69 ± 0.57	8.1
Ala	5.05 ± 0.52	8.5
Val	3.80 ± 0.60	3.9
Leu	9.65 ± 0.20	7.3
Ile	4.22 ± 0.18	1.4
Phe	2.80 ± 0.38	1.7
Tyr	3.26 ± 0.04	1.0
Trp	n.a.	1.4
Lys	6.92 ± 1.51	8.4
His	1.14 ± 0.11	1.9
Arg	4.30 ± 0.51	6.0

* Each value is the average of three determinations ± SE.
‡ Chromogranin A data are taken from Cohn et al. (7).
n.a., not analyzed.

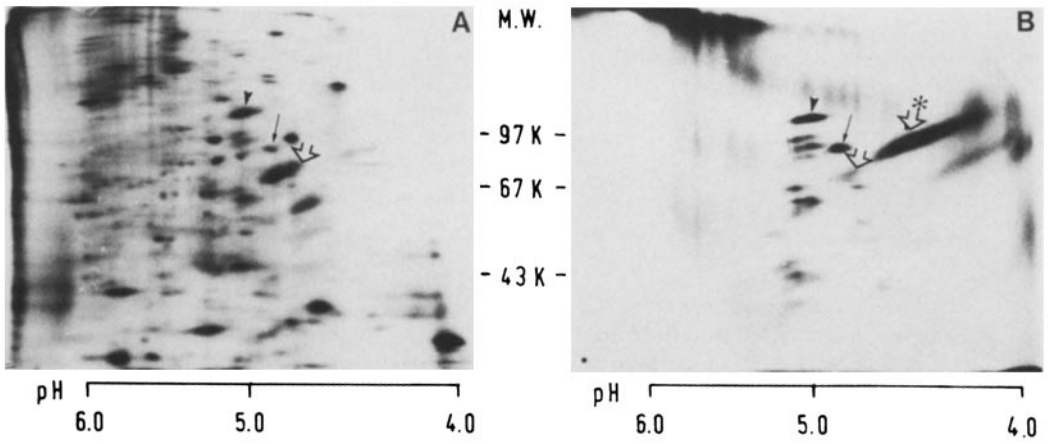

FIGURE 6 Phosphorylation and sulfation of secretogranin I, secretogranin II, and chromogranin A. [^{32}P]Phosphate-labeled (*A*) and [^{35}S]sulfate-labeled (*B*) proteins of adrenal medullary cells were separated by two-dimensional PAGE, followed by autoradiography (*A*) and fluorography (*B*). Arrowheads, secretogranin I; arrows, secretogranin II; open arrows, chromogranin A; open arrow with asterisk, highly sulfated form of chromogranin A. The identities of the radiolabeled proteins with secretogranin I, secretogranin II, and chromogranin A were determined by mixing and co-electrophoresis with the respective purified proteins.

TABLE II. *Tyrosine Sulfate Analysis of Secretogranin I, Secretogranin II, Chromogranin A, and Their Related Polypeptides*

[^{35}S]Sulfate-labeled material	^{35}S recovered after alkaline hydrolysis	^{35}S recovered as tyrosine sulfate
	cpm	*%*	*%*
Tyrosine [^{35}S]sulfate	2,340	80	66
Secretogranin I	1,395	52	42
p94	639	41	34
p90	594	51	52
p82	355	45	41
p67	450	52	47
Secretogranin II	1,413	53	41
p66	279	47	n.a.
Chromogranin A	391	8	n.a.
p92	1,813	2	n.a.

Total protein of [^{35}S]sulfate-labeled adrenal medullary cells was separated by two-dimensional PAGE. [^{35}S]Sulfate-labeled secretogranin I, secretogranin II, chromogranin A, and their related polypeptides, identified by mixing with unlabeled heat-stable chromaffin granule proteins, were excised from the gel and subjected to tyrosine sulfate analysis (21). Proteins were eluted under pronase digestion, and the eluates were subjected to alkaline hydrolysis using barium hydroxide followed by neutralization with sulfuric acid. The supernatant after neutralization was then analyzed by thin-layer electrophoresis. The ^{35}S recovered in the neutral supernatant after alkaline hydrolysis and in the tyrosine sulfate spot after thin-layer electrophoresis is expressed in percentage of the ^{35}S found in the pronase eluate.
n.a., not analyzed.

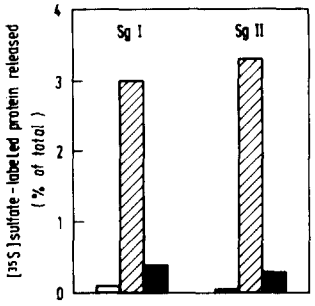

FIGURE 7 Depolarization-induced, calcium-dependent release of secretogranin I and secretogranin II from PC12 cells. [^{35}S]Sulfate-labeled PC12 cells were incubated in Ca^{2+}-containing medium in the presence of either 5 mM K^+ (▭), 55 mM K^+ (▨), or 55 mM K^+ plus 10 mM Mg^{2+} (■). [^{35}S]Sulfate-labeled secretogranins I and II released into the medium as well as those present in the cells were quantitated after immunoprecipitation and SDS PAGE. The release of secretogranin I (*SgI*) and of secretogranin II (*SgII*) is expressed as percent of the total (medium plus cells) secretogranin I and of the total secretogranin II, respectively.

incorporated into secretogranins I and II was recovered as tyrosine sulfate (Table II). This incomplete recovery and the tunicamycin result indicated the presence of O-linked sulfated oligosaccharide residues in addition to tyrosine sulfate residues in the two secretogranins. The various polypeptides related to secretogranins I and II and chromogranin A (see Figs. 2 and 4) were also phosphorylated and sulfated (Fig. 6, Table II).

Secretion of Secretogranins I and II

Depolarization of [^{35}S]sulfate-labeled PC12 cells resulted in the release of labeled secretogranins I and II into the medium (Fig. 7). The depolarization-induced release of secretogranins I and II was apparently calcium-dependent since it was blocked by the presence of 10 mM magnesium ions. This condition is known to inhibit depolarization-induced calcium-dependent exocytosis (10) and was found to block the depolarization-induced norepinephrine release (data not shown). [^{35}S]Sulfate-labeled secretogranins I and II, as well as chromogranin A, were released into the medium upon depolarization of primary cultures of bovine adrenal medullary cells (data not shown).

Occurrence of Secretogranins I and II in a Wide Variety of Endocrine and Neuronal Cells Secreting Peptides by the Regulated Pathway

In view of the presence of secretogranins I and II in anterior pituitary and adrenal medulla, we investigated the possible occurrence of the two secretogranins in other tissues containing endocrine cells and in the brain. For this purpose, the two proteins were enriched from various rat tissues by immunoprecipitation and their presence subsequently investigated by immunoblotting (Fig. 8).

Secretogranins I and II were detected in the brain, the duodenum, and the thyroid gland; secretogranin II was also detected in the pancreas. The relative proportion of secretogranin I to secretogranin II was different in the various tissues. Secretogranin II was predominant in the brain and thyroid, and secretogranin I was predominant in the duodenum. The levels of the two secretogranins in the submaxillary gland (Fig. 8) and in serum (data not shown) were below the limit of detection of the present immunoprecipitation/immunoblotting method. The latter finding indicated that the observed amounts of secretogranins in the various tissue homogenates could not be explained by the presence of blood containing secretogranins.

The occurrence of secretogranins I and II in a variety of rat tissues was further investigated by light microscopic immunocytochemistry (Figs. 9–12). Immunoreactivity for both or at least one of the two secretogranins was found in all of the various endocrine and neuronal tissues examined. In all cases,

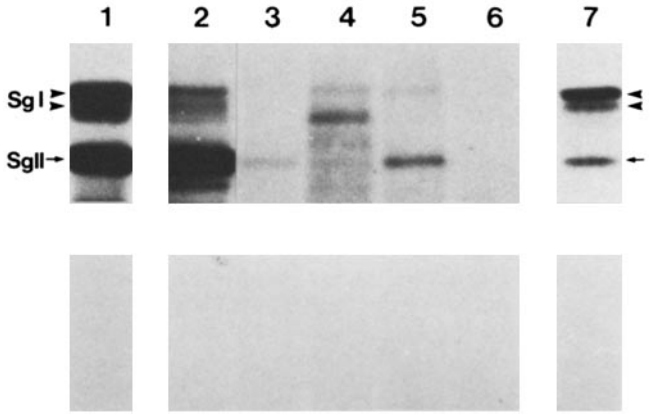

FIGURE 8 Presence of secretogranin I and secretogranin II in various tissues of the rat. Secretogranin I and secretogranin II were enriched from total homogenates of rat brain (4,320 μg protein, lane *2*), pancreas (4,165 μg protein, lane *3*), duodenum (3,885 μg of protein, lane *4*), thyroid (1,600 μg protein, lane *5*), and submaxillary gland (4,000 μg of protein, lane *6*) by immunoprecipitation, using a mixture of anti-rat secretogranin I antiserum and anti-rat secretogranin II antiserum. The immunoprecipitates and, as references, homogenates of rat pituitary (100 μg protein, lane *1*) and of PC12 cells (40 μg protein, lane *7*) were analyzed by one-dimensional immunoblotting, using a 1:1 mixture of either anti-rat secretogranin I antiserum and anti-rat secretogranin II antiserum (top) or the respective preimmune sera (bottom). Only the portions of the immunoblots containing secretogranin I (*SgI*) and secretogranin II (*SgII*) are shown. The exposure times of the fluorograms of lanes *1* and *7* and lanes *2–6* were 4 h and 30 h, respectively.

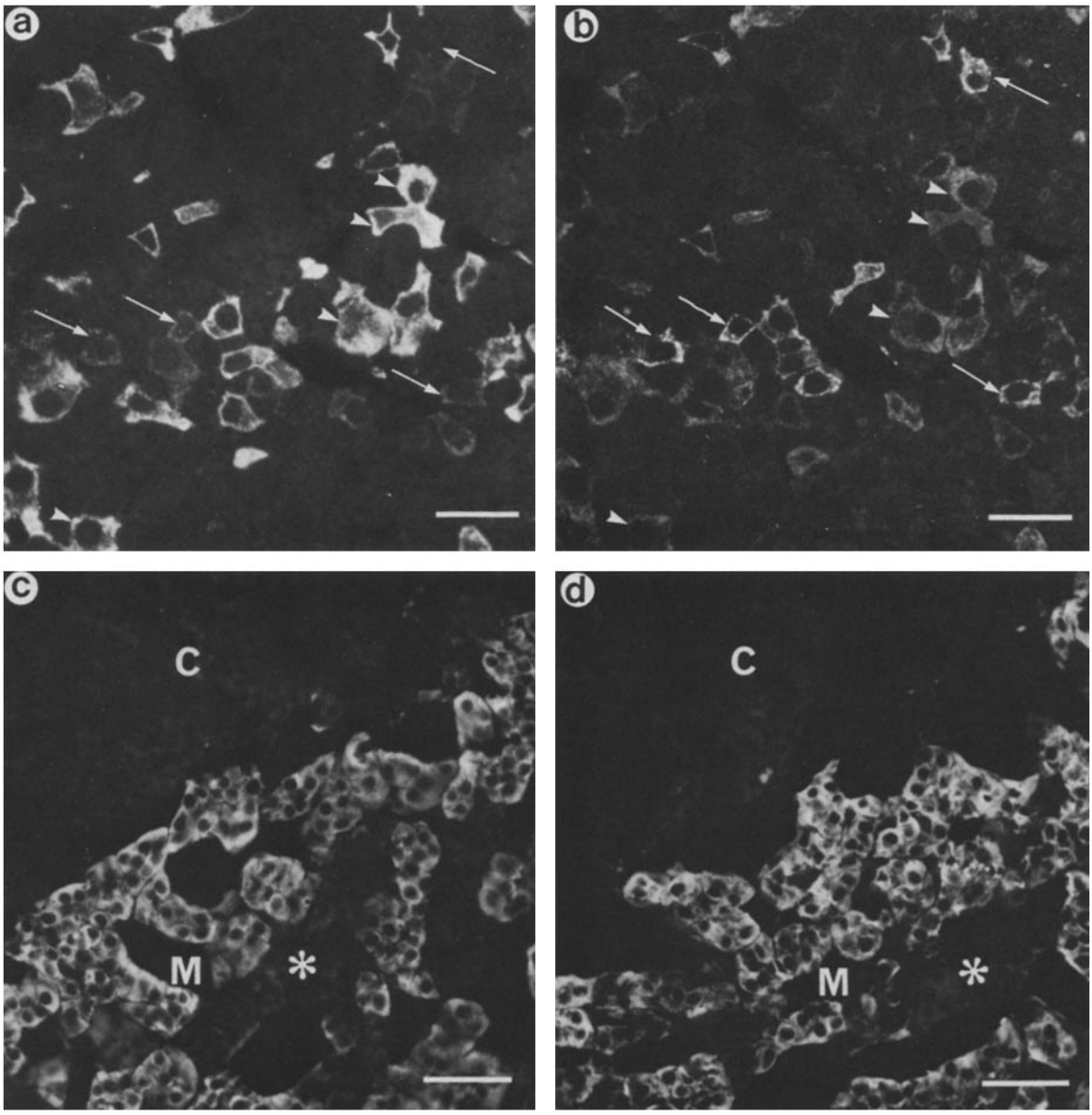

FIGURE 9 Presence of secretogranin I and secretogranin II in the rat anterior pituitary and adrenal gland as revealed by immunofluorescence. (*a* and *b*) Serial plastic sections (1-μm thick) of rat anterior pituitary immunostained with anti-rat secretogranin I antiserum (1:10 dilution, *a*) and anti-rat secretogranin II antiserum (1:10 dilution, *b*). Many, but not all cells are stained by both antisera. Positive cells exhibit variable degrees of immunoreactivity. Some cells that contain high levels of secretogranin I (arrowheads, *a*) contain only low levels of secretogranin II (arrowheads, *b*). Some cells that contain high levels of secretogranin II (arrow, *b*) contain only low levels of secretogranin I (arrows, *a*). (*c* and *d*) Frozen sections (20-μm thick) of rat adrenal gland immunostained with anti-rat secretogranin I antiserum (1:10 dilution, *c*) and anti-rat secretogranin II antiserum (1:10 dilution, *d*). Most cells of the medulla (*M*) but not cells of the cortex (C) are brightly immunostained for both secretogranins. Groups of poorly stained chromaffin cells are indicated by asterisks. (*a* and *b*) Bars, 20 μm. × 600. (*c* and *d*) Bars, 50 μm. × 256.

the immunoreactivity exhibited a punctate appearance (visible at higher magnification) consistent with the localization of these proteins in secretory granules (compare reference 38).

In line with the previously reported distribution of secretogranin II in the bovine anterior pituitary (38), this protein was found to be present, at variable concentration, in many but not all cells of the rat anterior pituitary. Secretogranin I was found to have a similar distribution, and most cells unstained by anti-secretogranin II antiserum were also unstained by anti-secretogranin I antiserum (Fig. 9, *a* and *b*). In positive cells, the relative proportion of secretogranin I and secretogranin II immunoreactivity varied. Staining of adjacent 1-μm-thick plastic sections revealed that many cells that were only moderately stained for secretogranin I were heavily stained for secretogranin II, and vice-versa.

In the adrenal gland, cells of the medulla but not of the cortex were immunoreactive (Fig. 9, *c* and *d*). Not all chromaffin cells, however, contained the same amount of secretogranins. Two subpopulations of chromaffin cells could be identified with both anti-secretogranin I and anti-secretogranin II antisera, one with a lower and one with a higher content of secretogranins.

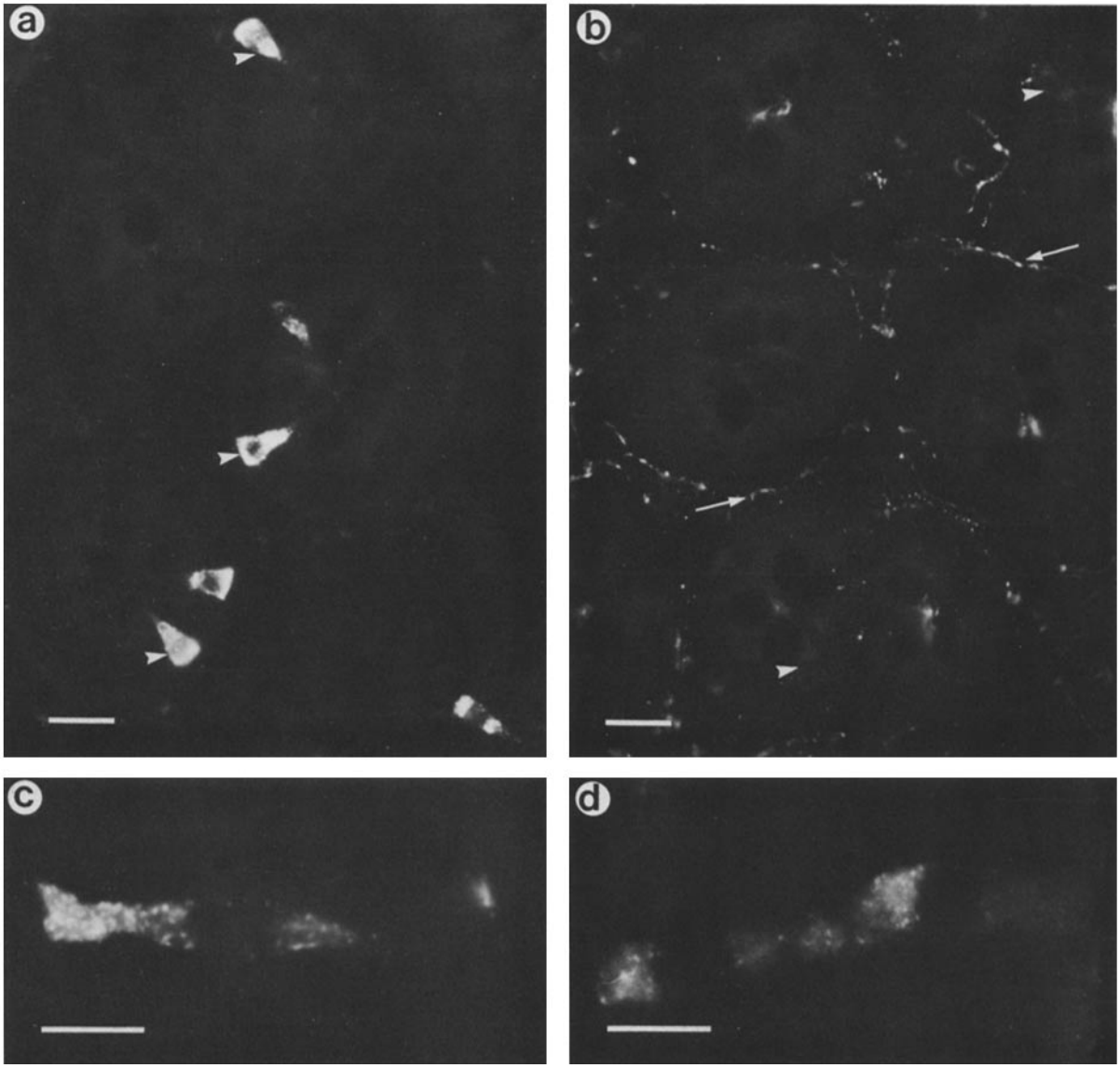

FIGURE 10 Presence of secretogranin I and secretogranin II in the rat duodenum as revealed by immunofluorescence. 10-μm-thick frozen sections immunostained with anti-rat secretogranin I antiserum (*a* and *c*) and anti-rat secretogranin II (*b* and *d*) antiserum. Secretogranin I immunoreactivity is concentrated in, and confined to, cells scattered in the mucosal epithelium, which have the characteristics of the typical endocrine cells of the gut (arrowheads, *a*). Secretogranin II can also be detected in some of these cells, but is present in very low levels (arrowheads, *b*). In addition, secretogranin II is concentrated in neuronal varicosities (arrows, *b*). *c* and *d* show higher magnifications of immunoreactive mucosal cells illustrating the punctate appearance of the immunostain. *d* has been printed at much higher contrast than *c* to better visualize the staining pattern, (*a* and *b*) Bars, 20 μm. × 461. (*c* and *d*) Bars, 10 μm. × 1,440.

Both secretogranins were detectable in the scattered endocrine cells of the mucosa in all portions of the stomach (data not shown) and duodenum (Fig. 10). The high number of immunoreactive cells suggested that most, if not all, endocrine cells were stained, irrespective of the type of regulatory peptide secreted. In the wall of the duodenum, immunoreactivity for the two secretogranins showed remarkable differences in distribution. In the mucosal endocrine cells of the duodenum, secretogranin I immunoreactivity was much more prominent than secretogranin II immunoreactivity. Secretogranin II was also detectable in neuronal elements throughout the intestinal wall (Fig. 10). Neurons of the Auerbach and submucosal plexus, as well as varicosities throughout the muscular, submucosal, and mucosal layers were stained. No secretogranin I immunoreactivity was observed in neuronal elements, even in sections that contained heavily stained endocrine mucosal cells.

In the pancreas, low levels of secretogranin II but not of secretogranin I were seen in the endocrine islands (not shown). In addition, stained axonal varicosities were visible throughout the tissue. In the thyroid, secretogranin II was detectable in parafollicular cells and in axonal varicosities (not shown).

Immunoreactivity for both secretogranins was widespread throughout the central nervous system and was very prominent in certain regions. In all regions, the distribution of immunoreactivity suggested a localization of secretogranins in neuronal processes and/or perikarya. Low power views of immunoperoxidase-stained coronal sections of the brain (Fig. 11) revealed that both secretogranins were more concentrated in the phylogenetically older portions of the brain, i.e., those

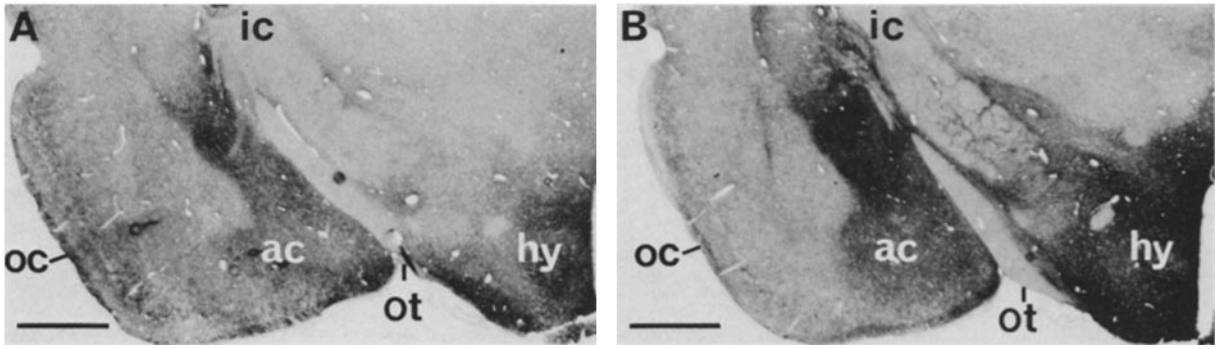

FIGURE 11 Presence of secretogranin I and secretogranin II in the ventral regions of the rat brain as revealed by immunoperoxidase. 15-μm-thick frozen coronal sections immunostained with anti-rat secretogranin I antiserum (*A*) and anti-rat secretogranin II antiserum (*B*). Peroxidase reaction product, which appears black in the micrographs, is concentrated in several areas that include the hypothalamus (*hy*) and the amygdaloid complex (*ac*). Note differences between distribution of immunoreactivity for secretogranin I (*A*) and secretogranin II (*B*). *ot*, optic tract; *oc*, olfactory cortex; *ic*, internal capsule. (*A* and *B*) Bars, 1 mm. × 12.1.

known to be most enriched in peptide neurotransmitters (24). Areas more enriched in secretogranins included the hypothalamus, the amygdala, the caudate-putamen, the olfactory cortex, and, in particular, the mossy fiber system of the hippocampus (Fig. 12). Inside major brain regions, the distribution of the two secretogranins was not identical. Clear differences were visible, for example, in ventral portions of the brain (Fig. 11). Differences were even more evident when individual brain regions were examined at higher magnification (not shown). The meshwork of processes that were immunoreactive for secretogranins I and II often had different patterns. In addition, the relative proportion of stain on perikarya and cell processes was also variable. For example, in some neocortical areas, immunoreactivity for secretogranin I was more concentrated in perikarya, while that for secretogranin II was more concentrated in fine varicose processes.

DISCUSSION

The present study reports on a major feature of most endocrine and neuronal cells that secrete peptides by the regulated pathway: these cells contain, in addition to the specific peptide hormone or neuropeptide secreted, at least one member of the secretogranin/chromogranin class of proteins. It is the presence of this class of proteins rather than the presence of any one member which marks these cells. Three members of this protein class, which are not necessarily the only ones existing, are known to date: (*a*) chromogranin A, which was first characterized in the adrenal medulla (for reviews see references 44 and 45); (*b*) secretogranin II, which was first characterized in the anterior pituitary (36, 48); and (*c*) secretogranin I, which was first characterized in PC12 cells (27). (The latter protein has also been called chromogranin B because of its presence in chromaffin granules (12, 15, 46). However, the name chromogranin B has also been used to refer to a degradation product of chromogranin A [34].)

Secretogranin I, Secretogranin II, and Chromogranin A Are Distinct Proteins

Our results establish that secretogranin I, secretogranin II, and chromogranin A are distinct, and exclude the possibility that they are related to each other by a precursor-product relationship. Antisera against either secretogranin I, secretogranin II, or chromogranin A can be raised which specifically recognize the respective antigen and its proteolytic fragments. Moreover, the fingerprints of the three proteins are clearly different and suggest that the individual mRNAs existing for the three proteins (Fig. 5; references 12 and 39) must code for, on the whole, distinct protein sequences. Nevertheless, the three proteins seem to contain at least a few homologous segments (40), which would explain why we have sometimes observed cross-reactivities between antisera raised against Coomassie Blue–stained antigens (unpublished data). The three proteins are differentially expressed in various cells. For example, secretogranin II but not secretogranin I is present in axonal varicosities of the duodenum (Fig. 10). In PC12 cells, secretogranin I- and II-mRNAs, but little if any chromogranin A-mRNA, are found (Fig. 5). It will therefore be important to determine whether the distinct mRNAs for secretogranins I and II and chromogranin A and the different levels of these mRNAs in various cells result from cell-specific, extensive alternating splicing of a single primary transcript or from cell-specific expression of different genes.

Secretogranin I, Secretogranin II, and Chromogranin A Belong to One Class of Proteins

Although secretogranin I, secretogranin II, and chromogranin A are distinct proteins, they have three key features in common, suggesting that they exert the same function. First, all three proteins are very acidic, with pI values between 4.2 and 5.2. They are not only rich in acidic amino acids but are also posttranslationally modified by addition of strongly acidic sulfate and phosphate groups. The proteolytic fragments of the three proteins have pI values very similar to the parent proteins, as if acidic groups were spaced at repetitive intervals in the molecules.

Second, all three proteins are secretory. After their synthesis by membrane-bound polysomes of the rough endoplasmic reticulum, they pass through the Golgi complex where they become phosphorylated and sulfated, and are then specifically sorted into secretory granules. All three proteins, as well as proteolytic fragments of these proteins arising during intracellular transport and storage, are released upon appropriate stimulation of secretion.

Third, secretogranins I and II, like chromogranin A (8, 33, 41), selectively occur in a wide variety of endocrine and neuronal cells secreting peptides by the regulated pathway. Although each of these three proteins has a wide distribution, none occurs in all peptide-secreting endocrine and neuronal cells. However, the distribution of these proteins is not only

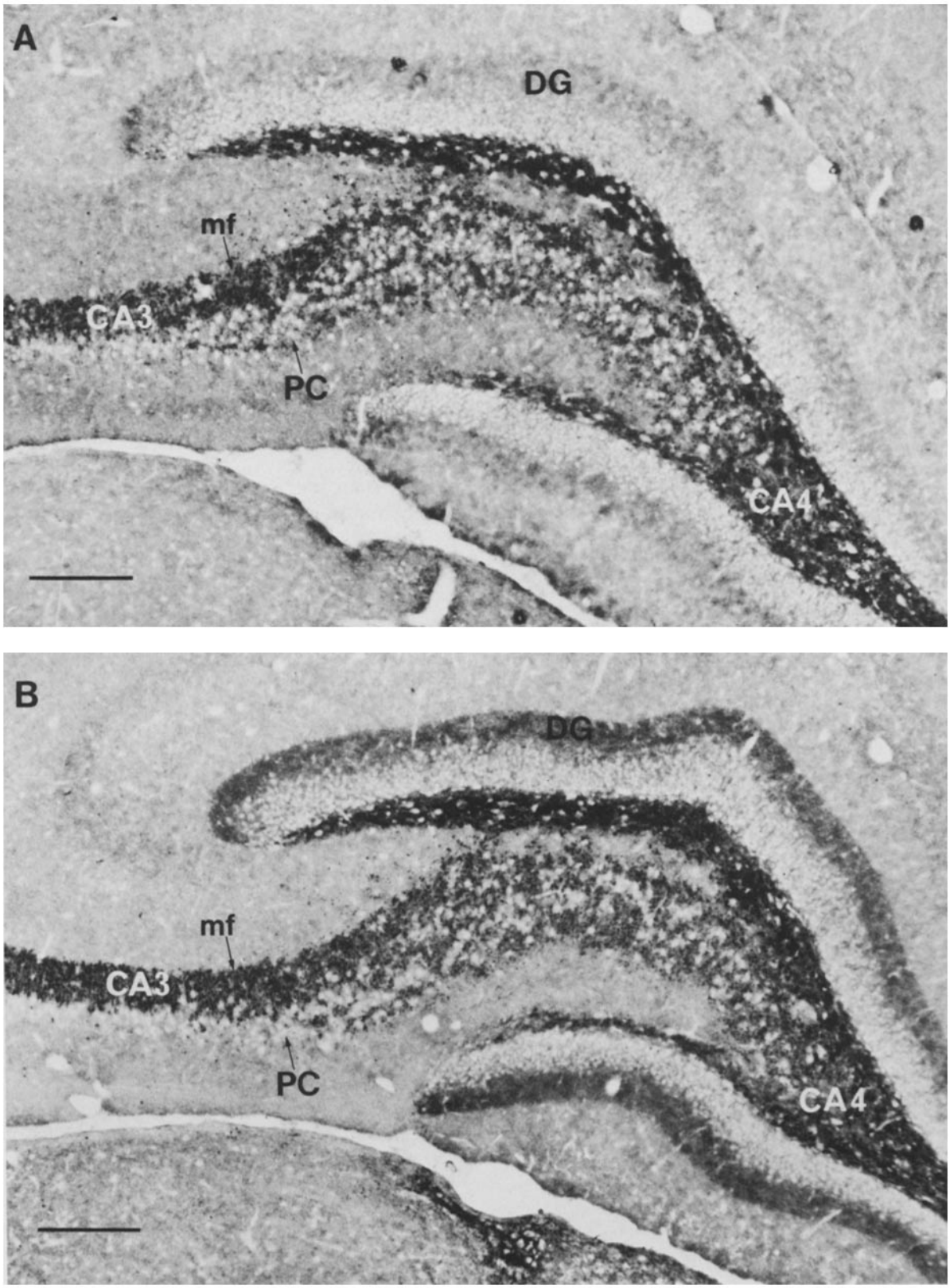

FIGURE 12 Presence of secretogranin I and secretogranin II in the mossy fiber system of the rat hippocampus as revealed by immunoperoxidase. 15-μm-thick frozen coronal sections stained with anti-rat secretogranin I antiserum (*A*) and anti-rat secretogranin II antiserum (*B*). The hylus of the hippocampus with the dentate gyrus (*DG*) and the CA4-CA3 region of the pyramidal cell layer (*PC*) are shown. Peroxidase reaction product, which appears black in the micrographs, is visible as punctate staining highly concentrated at sites that correspond in distribution to mossy fiber terminals (*mf*). (*A* and *B*) Bars, 200 μm. × 86.4.

overlapping but also complementary, as illustrated in this study for secretogranins I and II. Therefore, the distribution of this class of proteins is more widespread than that of any one of its members. The three proteins are not detected in cells secreting peptides by nonregulated pathways, in endocrine cells secreting nonpeptide hormones, and do not appear to be present in exocrine cells.

Relationship of the Secretogranin/Chromogranin Class of Proteins to Sulfated Proteoglycans of Secretory Granules

Chromaffin cells (see the open arrow with asterisk in Fig. 6) and anterior pituitary cells (data not shown) contain a highly sulfated, tunicamycin-resistant component. This component appears to be a highly sulfated form of chromogranin A (Figs. 2 and 4). Interestingly, this highly sulfated component also appears to correspond to one of the sulfated proteoglycans known to exist in chromaffin granules (23) since it can be digested with chondroitinase ABC (13). Together, these results suggest that the core protein of this proteoglycan is related to chromogranin A and raise the possibility of an interrelationship between sulfated proteoglycans of secretory granules and the secretogranin/chromogranin class of proteins.

Possible Functions of the Secretogranin/Chromogranin Class of Proteins

Proposals for the as yet unknown function of secretogranin I, secretogranin II, and chromogranin A should be reconciled with the specific occurrence of this class of proteins in secretory granules of a wide variety of endocrine and neuronal cells secreting peptides by the regulated pathway. It may be that these proteins function after secretion, for example as carrier proteins for regulatory peptides or as regulatory proteins themselves. One may also think of a role of these proteins in the secretory process of regulatory peptides, for example as processing enzymes or as helper proteins in the packaging of regulatory peptides into secretory granules (38). The latter role, although not proven, seems particularly attractive since it can be well reconciled with both the highly acidic nature of these proteins and the apparent relationship of at least one of these proteins, chromogranin A, to sulfated proteoglycans of secretory granules. The latter macromolecules have previously been proposed to be involved, by means of their high density of negative charges, in the packaging of proteins into secretory granules (14, 47). The secretogranin/chromogranin class of proteins would be similar to sulfated proteoglycans in that they, too, will carry a large number of negatively charged residues at the acidic pH of the *trans* cisternae of the Golgi complex (2), the compartment with a key role in the packaging of regulatory peptides.

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