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Distribution of Chromogranin A and Secretogranin I (Chromogranin B) in Neuroendocrine Cells and Tumors

RICARDO V. LLOYD, MARIA CANO, PATRIZIA ROSA, ANNETTE HILLE, and WIELAND B. HUTTNER

From the Department of Pathology, University of Michigan, Ann Arbor, Michigan, and European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany

The distribution of chromogranin A and secretogranin I (chromogranin B) in normal and neoplastic human endocrine tissues was analyzed with two human monoclonal antibodies against chromogranin A, anti-bovine antiserum against chromogranin A, and an anti-rat antiserum against secretogranin I. Western blotting analyses showed both chromogranin A and secretogranin I in normal adrenals, pheochromocytomas, a pituitary adenoma, and in normal pituitary glands, but not in a bladder carcinoma. Rat adrenal medullary and anterior pituitary tissues reacted with the polyclonal chromogranin A and secretogranin I antisera, but not with the two monoclonal chromogranin A antibodies. All antibodies reacted with most of the neuroendocrine cells and tumors examined. Pituitary prolactinomas contained immunoreactive secretogranin I, but not chromogranin A. Analysis of the distribution of chromogranin A and secretogranin I in pancreatic islet cells showed that chromogranin A was found predominantly in the glucagon-producing A cells, whereas secretogranin I was present in less than 5% of islet cells. These results indicate that chromogranin A and secretogranin I are both useful in the characterization of some neuroendocrine cells and neoplasms. (Am J Pathol 1988, 130:296-304)

THE DISTRIBUTION of chromogranin A, a secretory protein found in secretory granules, in normal and neoplastic endocrine cells has been analyzed by many investigators recently.[1-10] These studies have shown that most cells that form part of the diffuse neuroendocrine system[11] express chromogranin A. Widespread distribution of chromogranin A in the brain has also been reported.[10,12] Differences in the distribution of chromogranin A immunoreactivity in specific cell types of the pancreatic islet have been reported by various investigators.[4,5,8,9,13,14] Cohn et al[9] found chromogranin A only in the somatostatin and pancreatic polypeptide-producing islet cells of the rat pancreatic islets using rabbit antisera raised against bovine chromogranin A. Several other groups found that the pancreatic glucagon-producing cells stained strongly for chromogranin A, whereas the insulin- and somatostatin-producing cells had only weak or absent staining using monoclonal antibodies against human chromogranin A[4,5,8,13] or antisera against bovine chromogranin A.[14]

A wisespread distribution in secretory granules of endocrine cells and neurons is not restricted to chromogranin A, but is characteristic for other proteins as well. Two such proteins, secretogranin I and secretogranin II, were initially characterized in rat PC12 cells[15,16] and in the bovine anterior pituitary,[17-19] respectively. These two proteins occur in a wide variety of endocrine cells and neurons.[20,21] Secretogranin I and secretogranin II are distinct from each other and from chromogranin A by several criteria.[21] However, the three proteins have many properties in common, which suggests that they belong to one protein class.[21]

Secretogranin I and secretogranin II are thought to be similar to chromogranin B and chromogranin C,

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Address reprint requests to R. V. Lloyd, MD, Department of Pathology, University of Michigan, 2G332 Box 0054, Ann Arbor, MI 48109.

respectively, which have recently been characterized in adrenal medulla[22-24] and have also been found in several endocrine tissues.[25-28] In an attempt to simplify the nomenclature of these proteins, it has been suggested to refer to secretogranin I/chromogranin B as chromogranin B and to secretogranin II/chromogranin C as secretogranin II.[29] However, concerning secretogranin I/chromogranin B, recent sequence data[30] raise the possibility that the relationship between secretogranin I and chromogranin B is more complex than previously assumed.[29]

Although the distribution of chromogranin A in human neuroendocrine tumors has been analyzed by various investigators,[2-5,8] the distribution of secretogranin I in human neuroendocrine tumors has not been systematically analyzed. In this report we use several antibodies to 1) analyze the distribution of secretogranin I in selected normal and neoplastic human neuroendocrine tissues, using a specific anti-rat secretogranin I antiserum,[21] and 2) compare the distribution of secretogranin I to that of chromogranin A, using a well-characterized monoclonal antibody against human chromogranin A (LK2H10), a polyclonal antiserum against bovine chromogranin A, and another monoclonal antibody (PHE5) against human chromogranin A.

Materials and Methods

Immunoblotting

Tissues from human normal adrenal medulla, 2 pheochromocytomas, normal pituitary, a hormone-inactive pituitary adenoma, a nonfunctional pancreatic endocrine tumor, and a bladder carcinoma were obtained from surgically resected specimens (tumors) and from autopsies performed within 4 hours after death (normal tissues) and frozen at −70 C. In most cases, tissues were homogenized in 10 volumes of ice-cold PBS, pH 7.2, in a Polytron homogenizer (two 10-second bursts). The protease inhibitors phenylmethylsulfonyl fluoride (1 mM), benzamidine (10 mM), N-ethyl maleimide (5 mM) and ethylenediaminetetraacetic acid (5 mM) were added to the homogenization buffer. The homogenate was centrifuged at 10,000*g* for 10 minutes, and the supernatant was used for gel electrophoresis and Western blotting. Eighty micrograms of proteins were electrophoresed in 16 × 14 × 0.150-cm slab gells with a 10–17% gradient gel or a 12.5% gel with the discontinuous sodium dodecyl sulfate (SDS) buffer system of Laemmli.[31]

In the case of the human pituitary adenoma and of rat PC12 cells, the tissue and cell pellet, respectively, were directly homogenized in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels. In the case of 1 human pheochromocytoma, the heat-stable protein fraction was prepared as described[21] before SDS-PAGE.

Proteins from SDS gels were transferred to 0.45-μ nitrocellulose paper (Bio-Rad, Richmond, Calif) by electrophoretic transfer with a Bio-Rad Transblotting Cell (Bio-Rad) by the method of Towbin et al.[32] Detection of the protein bands was done as previously reported[33] with slight variation in the previous procedure. A biotinylated IgG and avidin–biotin peroxidase complex (ABC) (Vector Laboratories, Inc., Burlingame, Calif) were used instead of peroxidase-conjugated IgG. The monoclonal antibodies LK2H10 and PHE5 were used at 10 μg/ml, whereas antisera raised against bovine chromogranin A and rat secretogranin I were used at 1 : 100 and 1 : 50 dilutions, respectively. Some of the immunoblots for secretogranin I were performed as previously described, with affinity-purified anti-rat secretogranin I antibodies[30] or anti-rat secretogranin I antiserum[21] as indicated in the legend to Figure 2.

Immunohistochemical Staining of Tissues

The ABC immunoperoxidase method was used as previously described.[4] Formalin-fixed paraffin-embedded sections of human tissues accessioned at the University of Michigan were cut at 4 μ, dewaxed, and then treated with H_2O_2–methanol (1.5%) for 15 minutes. After washing in phosphate-buffered saline (PBS), pH 7.2, and treating with suppressor serum for 10 minutes, the tissues were incubated with the primary antibody for 60 minutes, then washed in PBS, and incubated with biotin–IgG (Vector) for 30 minutes. After PBS washes and incubation in ABC (Vector) for 30 minutes, the tissues were treated with diaminobenzidine-HCl (20 mg/dl) with 0.05% H_2O_2, washed with distilled water, and counterstained with hematoxylin.

Normal adrenal glands from untreated rats and pituitaries from rats treated with diethylstilbestrol to produce prolactin cell hyperplasia[34] were fixed in formalin, embedded in paraffin, and examined for immunoreactivity along with the human tissues.

Antibodies

The following antibodies and antisera were used for immunohistochemical studies. Anti-chromogranin A (LK2H10)[4,8] was used as a 1 : 10 dilution of tissue culture supernatant. PHE5 obtained from Enzo Biochem (New York, NY) was used at 1 μg/ml. Both

monoclonal antibodies belonged to the IgG1 subclass. Antibovine chromogranin A antiserum (Immuno-Nuclear, Stillwater, Minn)[9] was used at a 1:200 dilution. Anti-rat secretogranin I antiserum[21] was used at a 1:200 dilution. Anti-rat secretogranin I antibodies affinity-purified from the latter antiserum[30] were used at 12 μg/ml. Rabbit anti-rat prolactin, used at a 1:5000 dilution, was from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK). Negative controls consisted of substituting normal rabbit serum for the antisera. Negative controls for monoclonal antibodies LK2H10 and PHE5 consisted of substituting a mouse IgG1 isotypic immunoglobulin (Miles Laboratories, Elkhart, Ind).

Results

Immunoblotting of Chromogranin A and Secretogranin I

Immunoblotting of chromogranin A (Figure 1) showed very similar patterns in the normal adrenal, pheochromocytoma, normal pituitary, and pancreatic endocrine tumor with monoclonal antibodies LK2H10 and PHE5 and with the anti-bovine chromogranin A antiserum. This consisted of several distinct immunoreactive protein bands between 66 and 75 kd and some less intensely stained protein bands at lower molecular weights between 45 and 66 kd. The bladder carcinoma proteins did not react with any of the antibodies. Immunoblotting with ^{125}I-protein A and anti-secretogranin I antibodies (Figure 2) detected the 113-kd/105-kd forms of rat secretogranin I in the PC12 cell extracts and the approximately 120-kd and 100-kd forms of human secretogranin I in a pheochromocytoma and a pituitary adenoma, respectively (Figure 2). The antibodies against secretogranin I did not recognize human chromogranin A or secretogranin II.

Rat Tissues

Rat adrenal medullary tissues were diffusely positive after staining with anti-rat secretogranin I and anti-bovine chromogranin A antisera. The affinity purified anti-rat secretogranin I antibodies produced better staining than the unpurified antiserum in all tissues. Staining of rat adrenal tissue with the monoclonal antibodies LK2H10 and PHE5 against human chromogranin A was negative (data not shown). The hyperplastic pituitary tissues stained positively with anti-rat secretogranin I. This antiserum stained 50–70% of the pituitary cells (Figure 3), whereas the anti-bovine chromogranin A antiserum stained less

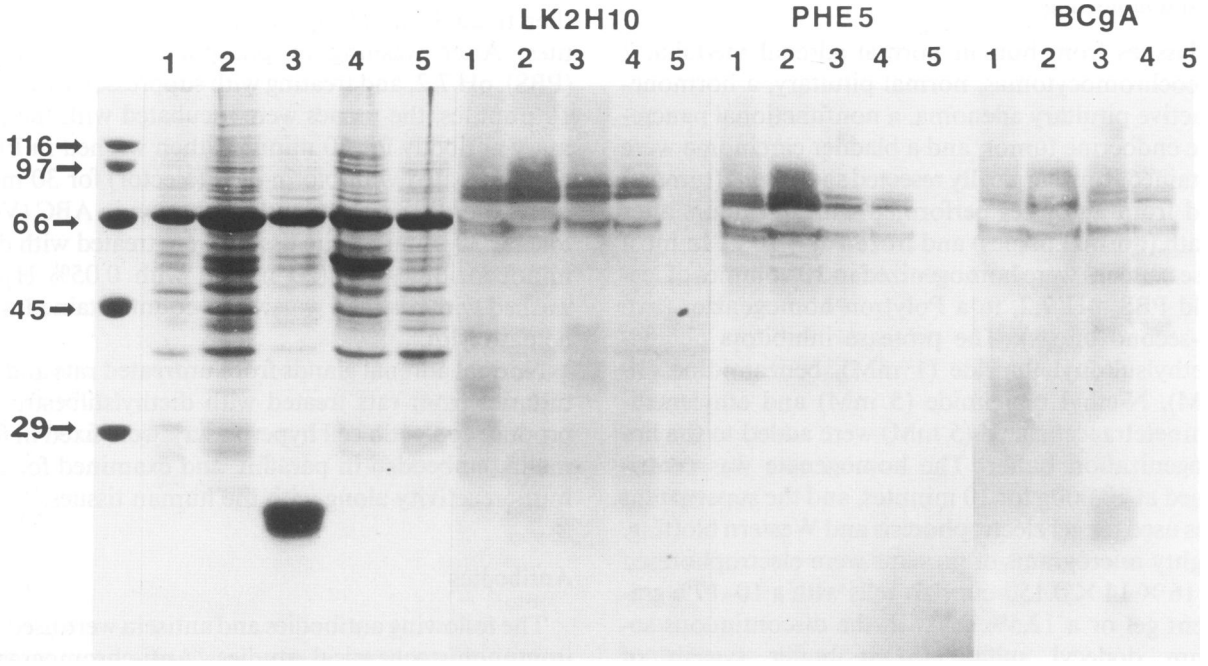

Figure 1—One-dimensional electrophoresis of normal and neoplastic human tissue homogenates. Homogenates were subjected to SDS-PAGE, and Coomassie blue staining was performed with the first panel on the left. Immunoblots were obtained with two monoclonal antibodies against chromogranin A (LK2H10 and PHE5) and an anti-bovine chromogranin A serum (BCgA). The following homogenates (80 μg) were examined: Lane 1, normal adrenal; Lane 2, pheochromocytoma; Lane 3, normal pituitary; Lane 4, nonfunctional pancreatic endocrine tumor; and Lane 5, transitional cell carcinoma of the bladder. Molecular weight standards are present in the first lane on the left.

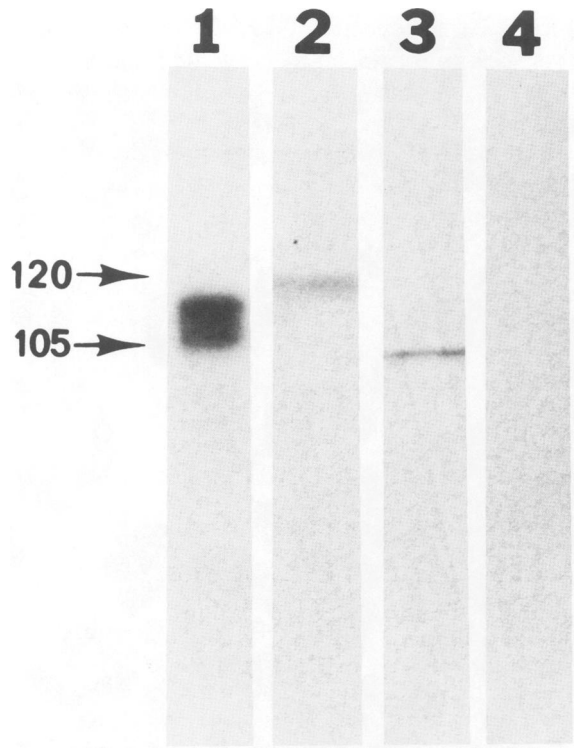

Figure 2—Autoradiogram showing results of SDS-PAGE followed by immunoblotting with ^{125}I-protein A and anti-secretogranin I antibodies. Lane 1, Total proteins (100 μg) of PC12 cells overlaid with 3 μg/ml affinity-purified secretogranin I antibody; Lane 2, heat-stable proteins (50 μg) of human pheochromocytoma overlaid with 6 μg/ml affinity-purified secretogranin I antibody; Lane 3, total proteins (200 μg) of a human pituitary adenoma overlaid with 1 : 200 diluted secretogranin I antiserum; Lane 4, same as Lane 3 overlaid with 1 : 200 diluted preimmune serum.

than 10% of the pituitary cells. Staining with anti-rat prolactin antiserum showed over 60% of cells positive for prolactin (Figure 3). Monoclonal antibodies LK2H10 and PHE5 did not react with rat pituitary or adrenal medulla.

Human Tissues

Immunostaining of human endocrine tissues is summarized in Table 1. In general, staining of normal and neoplastic endocrine tissues was similar. Normal adrenal medulla and paraganglionic tissues as well as pheochromocytomas were positive with all four antibodies (Figure 4). Many cells in the normal pituitary were positive for secretogranin I. The antiserum against secretogranin I stained most prolactinomas, whereas the other antibodies did not immunostain these tumors (Figure 5). Null cell (undifferentiated) adenomas and one gonadotropin-producing adenoma were positive for both chromogranin A and secretogranin I.

Immunostaining of the pancreatic islet for chromogranin A was most intense in cells around the periphery of the islets, which corresponded to the glucagon-producing A cells. Focal staining for secretogranin I was seen in some islet cells, but the positive cells, which constituted less than 5% of islet cells, were usually centrally located (Figure 6). Pure insulinomas (4 cases) were consistently negative with secretogranin I, whereas 1 of 4 cases was diffusely positive with the other three antibodies.

Medullary thyroid carcinomas, normal parathyroids, and parathyroid adenomas showed variable positive immunostaining for chromogranin A with all antibodies. These tissues and tumors were also positive for secretogranin I. Staining of the parathyroid adenomas was usually focal and less intense than in the normal parathyroid tissues. Parathyroid gland tissues stained less intensely for secretogranin I than for chromogranin A. The 1 case of a Merkel cell carcinoma was positive for chromogranin A with monoclonal antibodies LK2H10 and PHE5 but not with the anti-bovine chromogranin A antiserum. This tumor was also negative for secretogranin I.

Specificity of Immunostaining

Monoclonal antibodies against chromogranin A (LK2H10 and PHE5) stained neuroendocrine tissues and tumors exclusively. Pancreatic acinar cells, a rhabdomyosarcoma, a malignant fibrous histiocytoma, a bladder carcinoma, 2 papillary thyroid carcinomas, and a colonic carcinoma were negative. Antibovine chromogranin A and anti-rat secretogranin I antisera did not stain adenocarcinomas or sarcomas. Adrenal cortical tissues associated with normal medullae and with pheochromocytomas were negative with all four antibodies.

Discussion

These studies indicate that both chromogranin A and secretogranin I are present in many normal and neoplastic tissues of the diffuse neuroendocrine system. Immunoreactivity with monoclonal antibodies against chromogranin A (LK2H10 and PHE5) was almost identical, and neither of these antibodies reacted with rat adrenal medullary or hyperplastic pituitary tissues.

Immunoblotting studies revealed that monoclonal antibodies LK2H10 and PHE5 both recognized chromogranin A with molecular weights between 66 and 75 kd. The immunoblots with the anti-bovine chromogranin A antiserum were also similar, whereas immunoblots with anti-secretogranin I antibodies re-

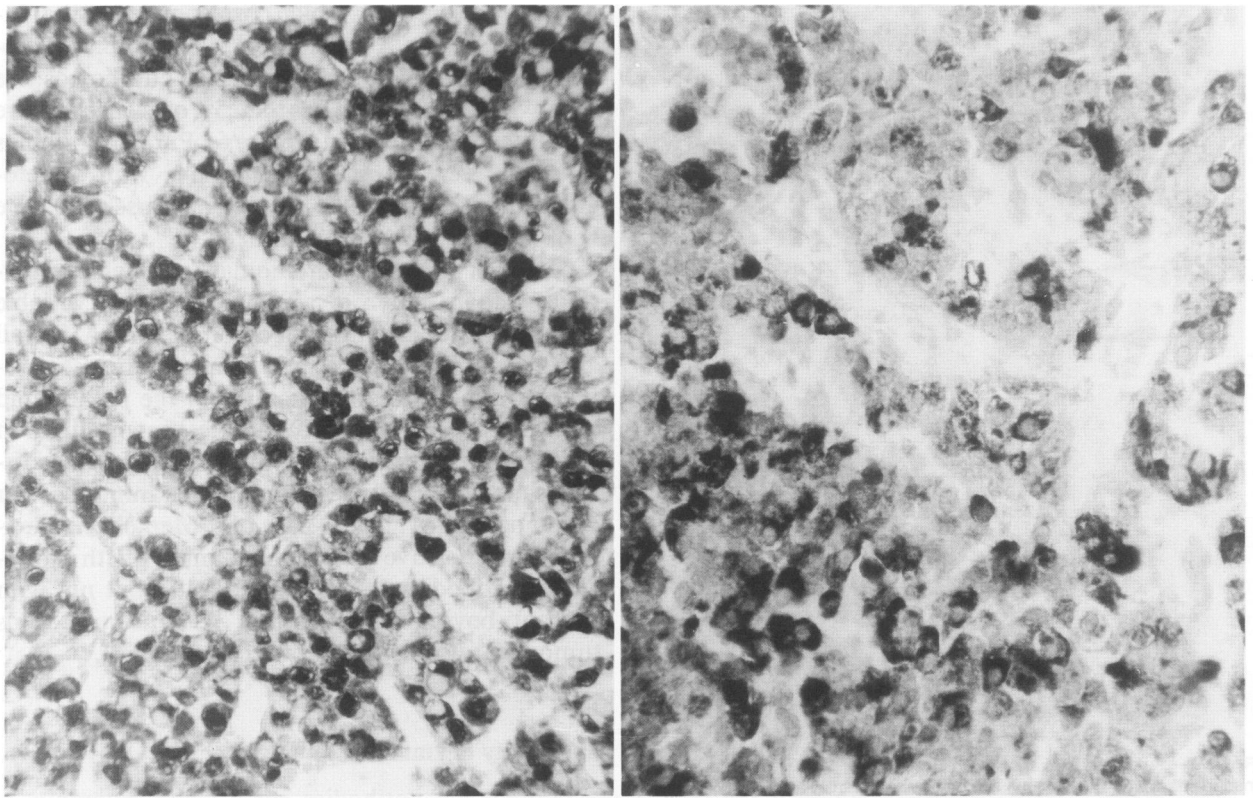

Figure 3—Hyperplastic pituitary from diethylstibestrol-treated rats. **A**—Staining with anti-rat prolactin antiserum shows positive immunoreactivity in many of the hyperplastic pituitary cells. (×330) **B**—Staining with anti-rat secretogranin I antibody also revealed many positive cells in the hyperplastic pituitary. (×330)

Table 1—Distribution of Chromogranin A and Secretogranin I in Human Endocrine Cells and Tumors

Diagnosis	n	Anti-CgA (LK2H10)	Anti-CgA (PHE5)	Anti-bovine CgA	Anti-SgI
Normal adrenal medulla	3	3	3	3	3
Normal paranganglion	1	1	1	1	1
Pheochromocytoma	4	4	4	4	4
Paraganglioma	3	3	3	3	3
Normal pituitary	3	3f	3f	3f	3f
Prolactinoma	7	0	0	0	5
Null cell adenoma	6	6	6	6	5
Gonadotropic adenoma	1	1	1	1	1
Normal islet	3	3f	3f	3f	3f
Insulinoma	4	1	1	1	0
Endocrine cells—duodenum	2	2	2	2	2
Gastrointestinal carcinoid	2	2	2	2	2
Pulmonary carcinoid	2	2	2	2	2
Normal parathyroid	2	2f	2	2f	2f
Parathyroid adenoma	3	3f	3	3f	3f
Medullary thyroid carcinoma	4	4	4	3	4
Merkel cell carcinoma of skin	1	1	1	0	0
Neuroblastoma	2	0	0	0	0
Esthesioneuroblastoma	1	1f	1f	1f	0
Small cell carcinoma of lung	4	0	0	0	0

Anti-CgAs (LK2H10 and PHE5) are monoclonal antibodies produced in mice against human pheochromocytomas.
Anti-bovine CgA is a rabbit polyclonal antiserum against bovine chromogranin A.
Anti-SgI is a rabbit polyclonal antiserum produced against rat PC12 secretogranin I.
f, focal staining, less than 25% of total cells.
n, number of cases studied.

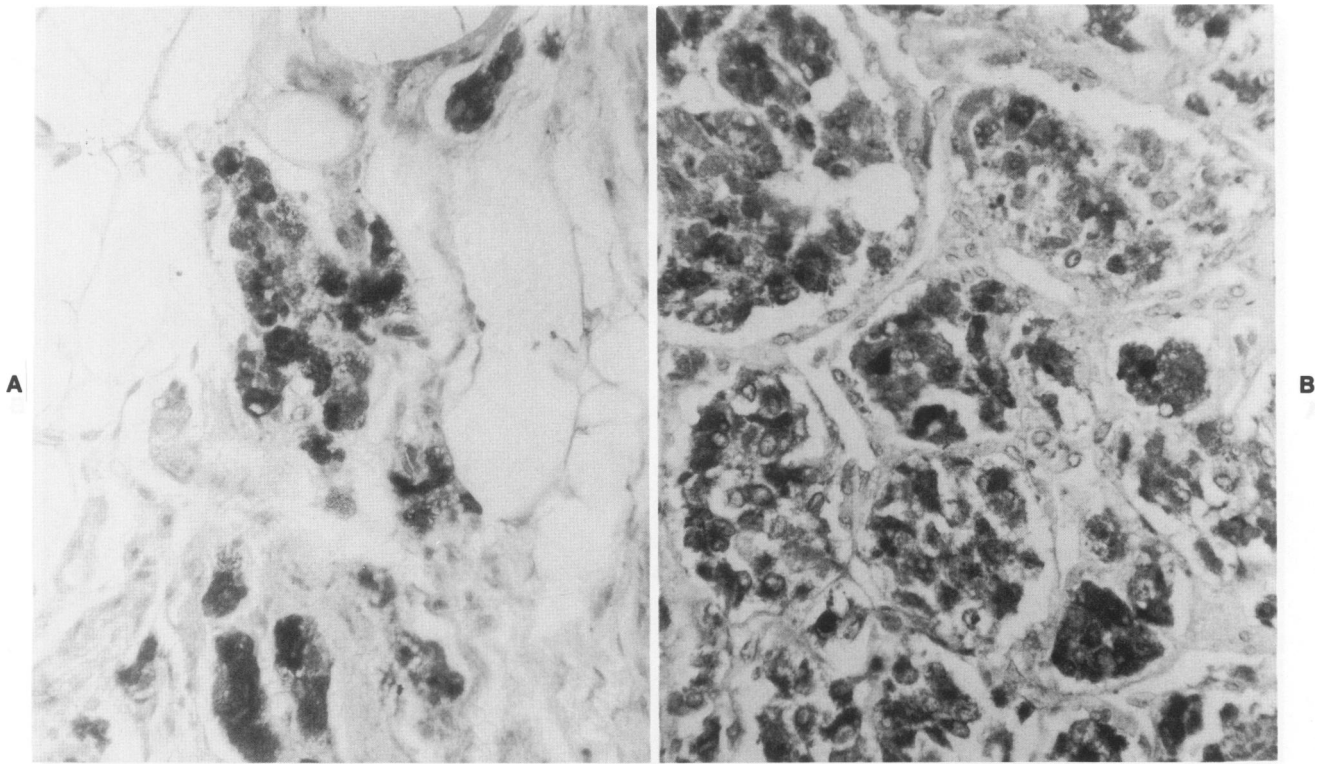

Figure 4—Immunochemical localization of secretogranin I in human chromaffin tissues. **A**—Normal paraganglionic tissue from the organ of Zuckerkandl stained strongly with rat anti-secretogranin I antiserum. (×330) **B**—Pheochromocytoma also shows diffuse immunostaining for secretogranin I. (×330)

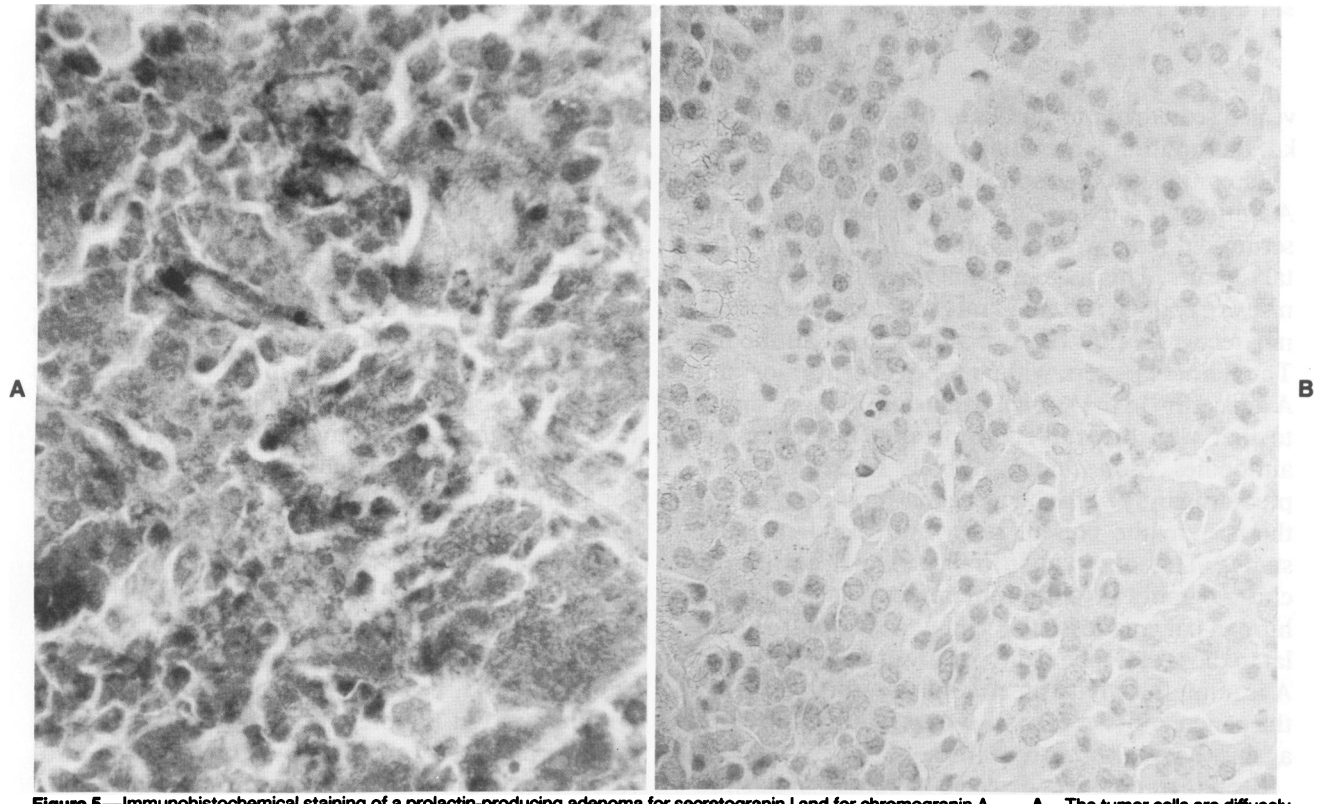

Figure 5—Immunohistochemical staining of a prolactin-producing adenoma for secretogranin I and for chromogranin A. **A**—The tumor cells are diffusely positive with anti-rat secretogranin I antiserum. (×330) **B**—Immunostaining of the tumor for chromogranin A with monoclonal antibody LK2H10 was negative. (×330)

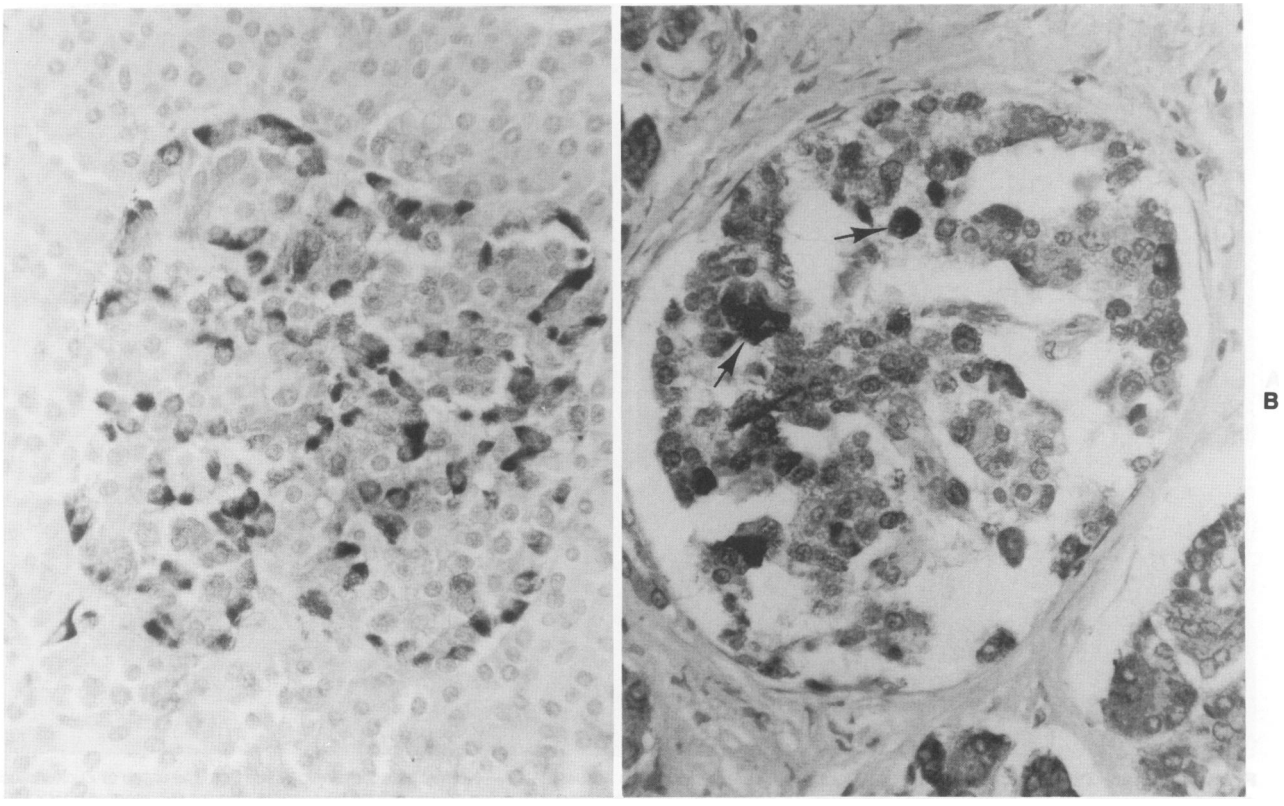

Figure 6—Localization of chromogranin A and secretogranin I in normal pancreatic islet. **A**—Immunostaining with monoclonal antibody LK2H10 directed against chromogranin A revealed intense staining predominantly in the cells located at the periphery of the islet. (×330) **B**—Immunostaining with anti-rat secretogranin I antiserum shows positive immunoreactivity in a small percentage of islet cells (*arrows*). (×330)

vealed secretogranin I proteins between 100 and 120 kd, but no immunoreactivity with chromogranin A.

In contrast to the monoclonal anti-chromogranin A antibody, the anti-bovine chromogranin A antiserum did react with rat adrenal medulla and pituitary tissues. This indicates that this antiserum recognizes an epitope present in rat chromogranin A that is not recognized by the two monoclonal antibodies. The staining pattern with anti-bovine chromogranin A antiserum in the human pancreatic islet was similar to that with the anti-chromogranin A monoclonal antibodies. Monoclonal antibody LK2H10 reacted predominantly with the glucagon-producing cells of the islet in the present study and in our previous study.[5] This observation indicates that anti-bovine chromogranin A antiserum reacts principally with human pancreatic islet A cells. Our findings are similar to those of Grube et al,[14] who found chromogranin A immunoreactivity predominantly in the A cells of the pancreatic islet of man and rat with an antiserum against bovine chromogranin A.

Secretogranin I was detected in human prolactinomas and in the normal human pituitary, whereas immunoreactive chromogranin A was not detected in prolactinomas with the use of three different anti-chromogranin A antibodies. These observations support our recent report indicating that prolactionomas did not express chromogranin A.[7] Rats treated with diethylstilbesterol, which produces prolactin cell hyperplasia, had secretogranin I immunoreactivity in many cells, whereas only a few cells in these hyperplastic pituitary, presumably gonadotropic cells,[7,9] were positive for chromogranin A with the bovine antiserum. Both monoclonal antibodies failed to detect chromogranin A-positive cells in the rat pituitary, whereas a few positive cells, probably representing gonadotropic cells,[7] were seen in human pituitaries. These findings suggest that secretogranin I, but not chromogranin A, is associated with secretory granules in prolactin-producing adenomas, and support earlier reports of Rosa et al[21] on the differential distribution of the members of the secretogranin/chromogranin protein class in secretory granules of different endocrine tissues. In the present study we also detected secretogranin I as well as chromogranin A immunoreactivities in normal parathyroid, in parathyroid adenomas, and in medullary thyroid carcinomas. However, anti-secretogranin I antiserum appears to

recognize different endocrine cells in the human islets of Langerhans than anti-chromogranin A antibodies.

Although chromogranin A was described in the adrenal medulla by several investigators many years ago,[35-38] the discovery of its similarity to secretory protein I of the parathyroid gland[1] and subsequent demonstration of the widespread distribution of chromogranin A in neuroendocrine cells and tumors[1-10] have increased interest in a search for the possible function of this molecule. O'Connor et al have shown that large amounts of chromogranin A are secreted by patients with endocrine tumors.[39,40] Recent immunohistochemical studies with antisera against secretogranin I and secretogranin II have supported the concept that these molecules may play a major role in the intracellular processing and possibly in the secretion of hormones.[21] A recent report indicated that porcine pancreastatin, a recently described peptide, was a potent inhibitor of insulin secretion.[41] The immediate recognition that this molecule was related to chromogranin A was made possible by the recent isolation and sequencing of cDNAs encoding bovine chromogranin A by various investigators.[42,43] The similarities in amino acid composition of pancreastatin to bovine chromogranin A raise the possibilities that pancreastatin may be produced from chromogranin A by limited proteolysis and that chromogranin A may function as a prohormone.[44,45] Another possible function that has been proposed for chromogranin A includes a role as a calcium regulatory protein.[46] Elucidation of the function(s) of the chromogranin/secretogranin proteins must await further studies.

The present findings indicate that secretogranin I and chromogranin A are present in many neuroendocrine cells and tumors, and that both markers can be used to characterize neuroendocrine cells and tumors, along with other broad spectrum neuroendocrine markers such as neuron-specific enolase and synaptophysin.[47,48]

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