

Synaptophysin: A marker protein for neuroendocrine cells and neoplasms

(synaptic vesicles/neurosecretory granules/pancreatic islets/carcinoids/tumor diagnosis)

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ABSTRACT Synaptophysin is an integral membrane glycoprotein (M_r 38,000) that occurs in presynaptic vesicles of neurons and in similar vesicles of the adrenal medulla. By using a monoclonal antibody to this protein (SY38), we have found, by immunohistochemistry and immunoblotting, that an identical or similar protein is also expressed in neuroendocrine tumors of neural type, such as pheochromocytomas and paragangliomas. In addition, this protein occurs in certain neuroendocrine epithelial cells, such as pancreatic islet cells; in a variety of neuroendocrine epithelial tumors, including islet-cell adenomas and carcinomas and several carcinoids and neuroendocrine carcinomas of the gastrointestinal and the bronchial tracts; and in medullary carcinomas of the thyroid. Our results show that synaptophysin, and the vesicles that contain it, can occur in normal and neoplastic neuroendocrine cells of neural type, as demonstrated by colocalization with neurofilaments, as well as in those of epithelial type, as shown by colocalization with cytokeratin filaments and desmoplakins. We conclude that synaptophysin is expressed independently of other neuronal differentiation markers and propose that it be used as a differentiation marker in tumor diagnosis.

Neuroendocrine cells comprise a widely distributed, morphologically and embryologically heterogeneous group (1, 2) present not only in the central and peripheral nervous systems but also in the gastrointestinal and bronchopulmonary tracts, the skin, the thyroid, and several other organs (3-5). The common property of these cells is the production of neurotransmitter substances such as acetylcholine, biogenic neuroamines, and neuropeptides (3-5). Numerous benign and malignant neoplasms either are entirely comprised of cells with neuroendocrine differentiation or may include a few such cells as part of complex populations (6). Normal, dysplastic, and neoplastic neuroendocrine cells may be identified by certain electron microscopic features, such as neurosecretory granules, or by the cytochemical demonstration of certain NE products (3-5). Immunocytochemical markers have proven especially important for the study of neuroendocrine differentiation, notably neuron-specific enolase, which is widely used as a broad marker for normal and neoplastic neuroendocrine cells (7-9), although the γ subunit of neuron-specific enolase is also found in a number of nonneuroendocrine cells (10). Neuropeptides can also be demonstrated in normal and neoplastic neuroendocrine cells and have proven valuable in tumor diagnosis (11, 12). However, as neuropeptides comprise a complex and variable group of substances, the application of a single peptide probe as a common marker of neuroendocrine differentiation is precluded (for discussion, see ref. 13).

The specific complement of cytoskeletal intermediate-filament (IF) proteins of neuroendocrine cells is also variable. Although neurons and certain other neuroendocrine cells express neurofilament proteins (NFP; refs. 14 and 15), many other neuroendocrine cells are clearly epithelial as defined by their expression of cytokeratin IF and desmosomal proteins (12, 16, 17). The corresponding neuroendocrine neoplasms express either NFP or cytokeratin filaments, respectively, but at least a few neuroendocrine neoplasms are capable of coexpressing both classes of IFs (13, 17-19).

Recently, an integral membrane glycoprotein (polypeptide M_r 38,000) has been identified in presynaptic vesicles (20, 21) and termed synaptophysin (21). By use of a monoclonal antibody, SY38, it has been shown (21) that this protein is specifically located in neuronal vesicles with an electron microscopically clear content and also occurs in the adrenal medulla of a number of mammalian species (see also ref. 20). Here we show that synaptophysin is present in a variety of human neuroendocrine cells and neoplasms of both the neural and the epithelial type.

MATERIALS AND METHODS

Tissues. Table 1 lists the neuroendocrine neoplasms examined and the pertinent pathologic and clinical data. For comparison, metastatic melanomas and a number of carcinomas of squamous and glandular differentiation lacking neuroendocrine features were studied. Tumor samples were frozen immediately after surgical removal or within 1 hr after death in the case of autopsy material (12, 21). Samples of normal human tissues were obtained from routine biopsies or autopsies. Various bovine tissues were similarly studied.

Light and Electron Microscopy. Samples were fixed either in Bouin's solution or in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin as well as with various special stains. For immunohistochemistry on paraffin sections, tissue fixed in Bouin's solution was preferentially used (23). Immunostaining was performed by the peroxidase-antiperoxidase technique (24) or by the avidin-biotin technique (25). Neuroendocrine substances examined are listed in Table 1; sources and dilutions of antibodies as well as methods and details on positive and negative controls have been described (12, 23).

Immunofluorescence microscopy on cryostat sections of frozen tissues (21) used the following antibodies: (i) murine monoclonal cytokeratin antibody PKK1 (ref. 26; from Labsystems, Helsinki, Finland); (ii) monoclonal antibody K_s 18.18, specific for cytokeratin no. 18 (16); (iii) guinea pig antibodies to cytokeratins (27); (iv) guinea pig antibodies to murine vimentin (28); (v) guinea pig antibodies against the

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Abbreviations: IF, intermediate-sized filament; NFP, neurofilament protein(s).

Table 1. Occurrence of synaptophysin in human neoplasms, as detected by immunocytochemistry with antibody SY38

Diagnosis (no. of cases)	Synap- tophy- sin	Cytoskeletal proteins				Neuroendocrine markers										Clinical syndrome	
		Cktn	NFP	Vmtn	Dpkn	NSE	ACTH	Bmbn	Cltn	Insl	Glg	[Leu]EK	Srtn	Smtn	SP		VIP
Adrenal pheochromocytomas (2)	2/2	0/2	2/2	0/2	0/2	2/2	0/2	1/2	1/2	0/2	0/2	1/2	1/2	0/2	0/2	2/2	Paroxysmal hypertension (2/2)
Paragangliomas (2)	2/2	0/2	2/2	0/2	0/2	2/2	0/2	1/2	2/2	0/2	0/2	1/2	1/2	0/2	1/2	1/2	None
Ganglioneuroblastomas (2)	2/2*	0/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	None
Islet cell microadenomas (1)	+	+	-	-	+	+	-	-	-	+	+	-	-	+	-	-	None
Islet cell adenoma (3)	3/3	3/3	2/3	0/3	2/3	3/3	0/3	0/3	0/3	3/3	3/3	0/3	0/3	2/3	0/3	0/3	Mild, episodic hypoglycemia (2/3)
Islet cell carcinoma (1)	+	+	-	-	+	+	-	-	-	+	+	-	-	+	-	+	Watery diarrhea
Carcinoids of lung (5)	3/5	5/5	3/5	-	5/5	5/5	0/5	3/5	3/5	0/5	0/5	2/5	2/5	0/5	0/5	0/5	None
Neuroendocrine carcinomas of lung (2)	2/2	2/2	0/2	0/2	2/2	2/2	0/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	None
Carcinoids of ileum (2)	2/2	2/2	0/2	0/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2	0/0	0/2	None
Neuroendocrine carcinoma of colon (1)	+	+	-	-	+	+	-	+	-	-	-	-	+	+	-	-	None
Medullary carcinomas of thyroid (3)	3/3	3/3	2/3	2/3	3/3	3/3	0/3	1/3	3/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	None
Neuroendocrine carcinomas of skin (5)	1/5	5/5	4/5	ND	ND	5/5	0/5	3/5	0/5	0/5	0/5	2/5	0/5	0/5	1/5	1/5	None

Cktn, cytokeratins (in particular nos. 8 and 18; see ref. 22); Vmtn, vimentin; Dpkn, desmoplakins; NSE, neuron-specific enolase; ACTH, corticotropin; Bmbn, bombesin; Cltn, calcitonin; Insl, insulin; Glg, glucagon, [Leu]EK, [Leu⁵]enkephalin; Srtn, serotonin; Smtn, somatostatin; SP, substance P; VIP, vasoactive intestinal peptide. ND, Not done.
*Synaptophysin restricted to some mature ganglion cells.

NFP polypeptide NF-H from porcine brain (21); (vi) monoclonal antibodies to neurofilament proteins NF-L and NF-M (ref. 29; from Boehringer Mannheim, F.R.G.); (vii) murine monoclonal antibodies DP1&2-2.15 and DP1-2.17 against desmoplakin (30); (viii) guinea pig antibodies to desmoplakins I and II (31); (ix) murine monoclonal antibody SY38 against synaptophysin (ref. 21; from Boehringer Mannheim).

Fluorescein isothiocyanate-labeled goat antibodies to guinea pig IgG (Bio-Yeda, Rehovot, Israel) and fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-labeled goat antibodies to murine immunoglobulin (Medac, Hamburg, F.R.G.) were used as secondary antibodies.

Electrophoresis and Immunoblotting. Tissue samples were used directly or homogenized in 2-3 volumes of ice-cold buffer (140 mM NaCl/2.6 mM KCl/6.4 mM Na₂HPO₄/1.4 mM KH₂PO₄/0.1 mM dithioerythritol/0.005% phenylmethylsulfonyl fluoride, pH 7.4) by 10 strokes with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 5000 × g for 10 min and the pellet was discarded. An aliquot of low-speed supernatant was used directly at different protein concentrations. Another aliquot was ultracentrifuged at 130,000 × g for 120 min, and the resulting pellet was designated "high-speed pellet." NaDodSO₄/PAGE and immunoblotting were as described (21).

RESULTS

Immunocytochemistry of Normal Tissues. Immunostaining of human brain, spinal cord, and muscle tissue with SY38 revealed intense reaction in neuronal structures, prominently

at the synapses, where it appeared as a finely punctate pattern (not shown; see ref. 21).

SY38 immunostaining of normal bovine and human fetal pancreas (Fig. 1), as well as normal-appearing regions of adult human pancreatic tissue (not shown), showed a specific and intense reaction of the epithelial complement of the islets of Langerhans, whereas the insular stroma as well as the exocrine parenchyma and stroma remained unstained. Immunostaining for cytokeratins, notably nos. 8 and 18 (22), showed less prominent, usually focal reaction in the islet cells, whereas the surrounding exocrine acini and ducts were strongly positive; staining for desmoplakins (31) displayed the typical coarsely punctate staining along plasma membranes in both exocrine and endocrine cells (not shown). NFP immunoreactivity was absent in the islet cells but present in the axons.

Immunohistochemistry of Tumors. When examined by electron microscopy, the diverse neuroendocrine tumors showed variable frequencies of dense-core neurosecretory granules (data not shown; refs. 4, 5, 12, 23). They also were reactive for neuron-specific enolase and one or more hormones (Table 1).

Neural Tumors. Immunofluorescence microscopy of pheochromocytomas and paragangliomas for synaptophysin and NFP revealed, in all cases, intense reaction within virtually all neoplastic cells for both kinds of proteins, whereas the fibrovascular stroma remained unstained. Fig. 2 presents as an example a pheochromocytoma. At higher magnification, the SY38 immunostaining appeared as a finely punctate pattern, whereas NFP was seen as fibrillar tangles

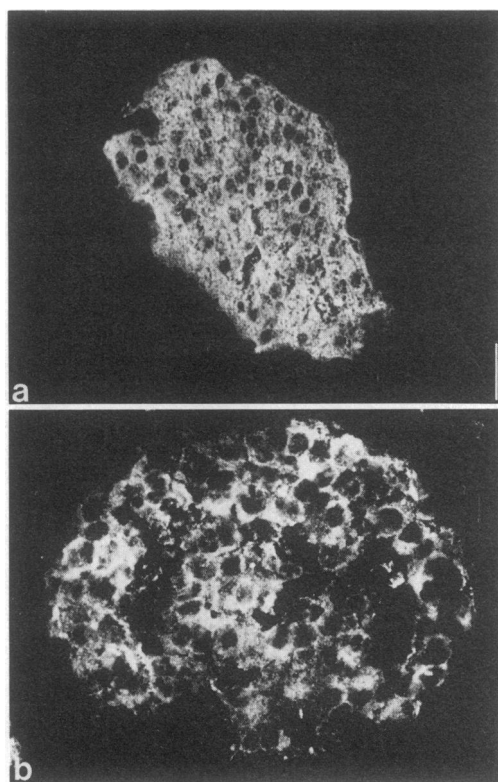


FIG. 1. Immunolocalization of synaptophysin in frozen sections of bovine (*a*) and human (*b*; 890-g fetus) pancreas, using antibody SY38. Immunoreaction is restricted to the islet cells. (Bars = 30 μ m.)

(Fig. 2 *c* and *d*). Cells with positive SY38 reaction were also found in ganglioneuroblastomas, medulloblastomas, and neuroblastomas (Table 1).

Epithelial Neuroendocrine Tumors. SY38 immunostaining of all pancreatic islet-cell neoplasms was intense and evenly distributed, irrespective of the variant of tumor, the hormone(s) produced, the number of neurosecretory granules, and the clinical manifestations (Table 1). The example shown in Fig. 3 is a vasoactive intestinal peptide-producing islet-cell carcinoma associated with a severe watery diarrhea syndrome. Double-label immunostaining for synaptophysin and the mesenchymal IF marker vimentin showed reaction with SY38 in the tumor parenchyma (Fig. 3*a*), whereas only the stroma was stained by vimentin antibodies (Fig. 3*b*). The tumor cells were also strongly positive for cytokeratins (Fig. 3*c*) and desmoplakins (Fig. 3*c* *Inset*). Immunoreaction for NFP was noted neither in the carcinoma nor in the stromal cells but was restricted to the sparse neural structures included in the tumor (Fig. 3*d*).

Most of the carcinoids and all of the medullary thyroid carcinomas examined were immunoreactive with SY38 (an example of an ileal carcinoid is shown in Fig. 4*a*), often revealing the characteristic, finely punctate pattern related to the vesicles (Fig. 4*b*). Cytokeratin antibodies stained fibrillar structures throughout the cytoplasm (Fig. 4*c*), and desmoplakin staining revealed the typical punctate arrays along cell boundaries (Fig. 4*d*). The correlation of immunostaining for cytokeratins and desmoplakins with that of synaptophysin showed that synaptophysin was present in the epithelial tumor cells.

We noted a similar staining pattern for synaptophysin not only in frozen sections, by immunofluorescence microscopy, but also in sections of paraffin-embedded specimens, using the avidin-biotin technique. Melanomas, including some expressing neuroendocrine markers, as well as variably

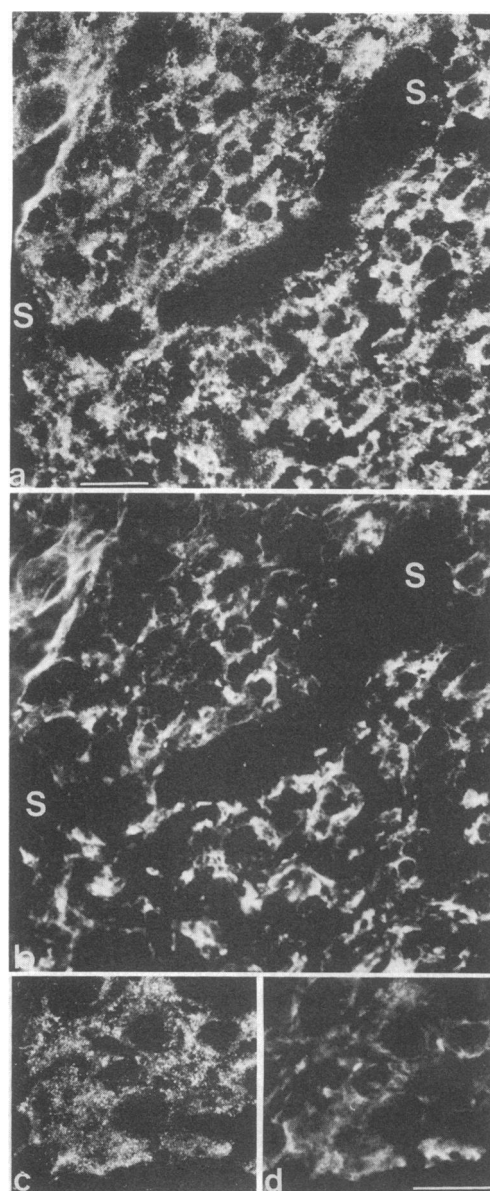


FIG. 2. Double-label immunofluorescence microscopy of frozen sections of human pheochromocytoma, using anti-synaptophysin antibody SY38 (*a* and *c*) and guinea pig antibodies to NFP (*b* and *d*). Tumor cells are positive for both proteins, but staining with SY38 (*a* and *c*) is scattered and punctate (for higher magnification see *c*), whereas NFP is concentrated in coarser clumps and fibrillar structures (*b* and *d*). Stroma (S) is negative. Micrographs *a* and *b* and micrographs *c* and *d* show the same sections, respectively. (Bars = 20 μ m.)

differentiated squamous and glandular, nonneuroendocrine carcinomas from diverse sites did not stain with SY38.

Identification of Synaptophysin by Immunoblotting. To identify the polypeptide immunoreactive with SY38 in normal tissues and tumors, proteins of cell fractions were examined by immunoblotting (Fig. 5). An immunoreactive polypeptide of M_r 38,000 was identified in the low-speed supernatant fractions and was enriched in the pellets from high-speed centrifugation, which contained a higher concentration of small vesicles. While the reactive polypeptides present in human brain (lane 2'), islet cell carcinoma (lanes 3' and 6'), ileal carcinoid, medullary carcinoma of thyroid (not shown), and pheochromocytoma (lanes 5 and 8') all showed the aforementioned M_r value, the paraganglioma contained an immunoreactive polypeptide of $M_r \approx 40,000$ (lanes 4' and 7'), perhaps reflecting a difference in glycosylation.

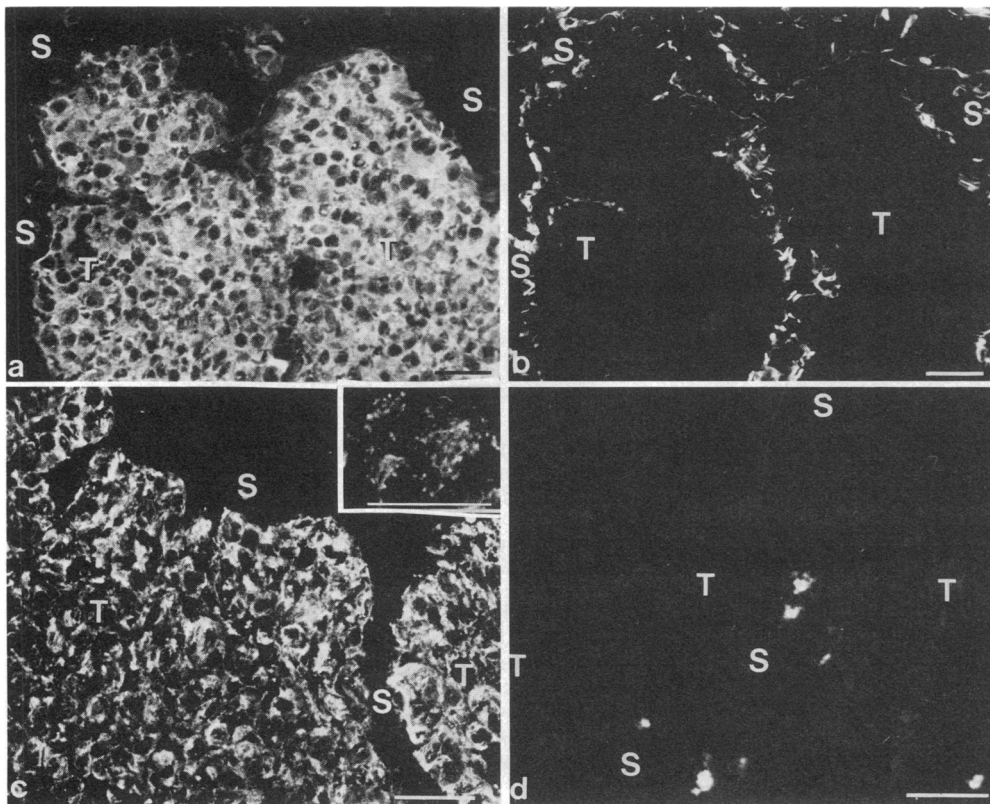


FIG. 3. Immunofluorescence microscopy of human vasoactive intestinal peptide (VIP)-producing islet-cell carcinoma, using antibody SY38 (*a*) and antibodies to vimentin (*b*; antibody from guinea pig), cytokeratins (*c*; antibody K, 18.18), desmoplakins (*Inset* in *c*; antibody DP1&2-2.15), and NFP (*d*; same antibody as in Fig. 2*b*). *a* and *b* show double-label immunostaining of the same section. Note specificity of SY38 reaction for tumor (T) cells, whereas vimentin is exclusive for stromal (S) cells. Tumor cells are positive for cytokeratins and desmoplakins (*c* and *Inset*). Tumor and stromal cells are negative for NFP, which is restricted to sparse neuronal structures included in the tumor (positively stained sites in *d*). (Bars = 30 μ m.)

DISCUSSION

We have demonstrated the presence of a vesicle membrane protein, synaptophysin, in neurons, adrenal medulla, pancreatic islet cells, and a series of neuroendocrine neoplasms. This shows that synaptophysin and the vesicles that contain it are not exclusive to neuronal differentiation but are also formed in certain epithelial cells, and that the synthesis of this protein is independent of the expression of other neural markers, notably NFP and neurosecretory products. Our findings also show that synaptophysin-containing vesicles continue to be formed during cell transformation and malignant growth.

The finding of synaptophysin in pheochromocytomas, paragangliomas, and the other neural tumors is not unexpected, given its presence in normal neurons, nor is the expression of this protein unexpected in pancreatic islet cell tumors, given its abundance in normal islet cells. Thus, in these tumors, synaptophysin represents a conserved differentiation marker. However, the occurrence of synaptophysin in many, though not all, carcinoids and neuroendocrine carcinomas, including medullary carcinomas of the thyroid, is more difficult to explain, as we have not detected this protein in all corresponding normal tissues. Therefore, we cannot exclude at present that, in some tumors, synaptophysin can be formed *de novo* in the process of neoplastic transformation.

We propose that antibodies to synaptophysin be used in the identification and characterization of neuroendocrine tumors as a novel type of neurosecretory marker—namely, an integral membrane component, whose expression is not dependent on the synthesis of other markers of neuroendocrine differentiation. Synaptophysin antibodies should also be useful in differential tumor diagnosis, in particular for the exclusion of malignant melanomas, which often present some neuroendocrine markers such as neuron-specific enolase and certain neuropeptides.

The presence of synaptophysin in a number of benign and malignant neuroendocrine neoplasms, and its absence in

other neuroendocrine tumors of the same category, raises the question of the possible heterogeneity of these tumors (5, 6). A similar situation has been described for another kind of neuroendocrine markers, the proteins associated with the contents of neurosecretory vesicles of the dense-core type, such as chromogranin A and secretogranins I and II, which are detectable in certain neurons and adrenal medullary and epithelial neuroendocrine cells but not in others (32–34). Hence, the presence of synaptophysin on the one hand and secretogranins on the other could provide important criteria to divide the various neuroendocrine tumors into different subtypes.

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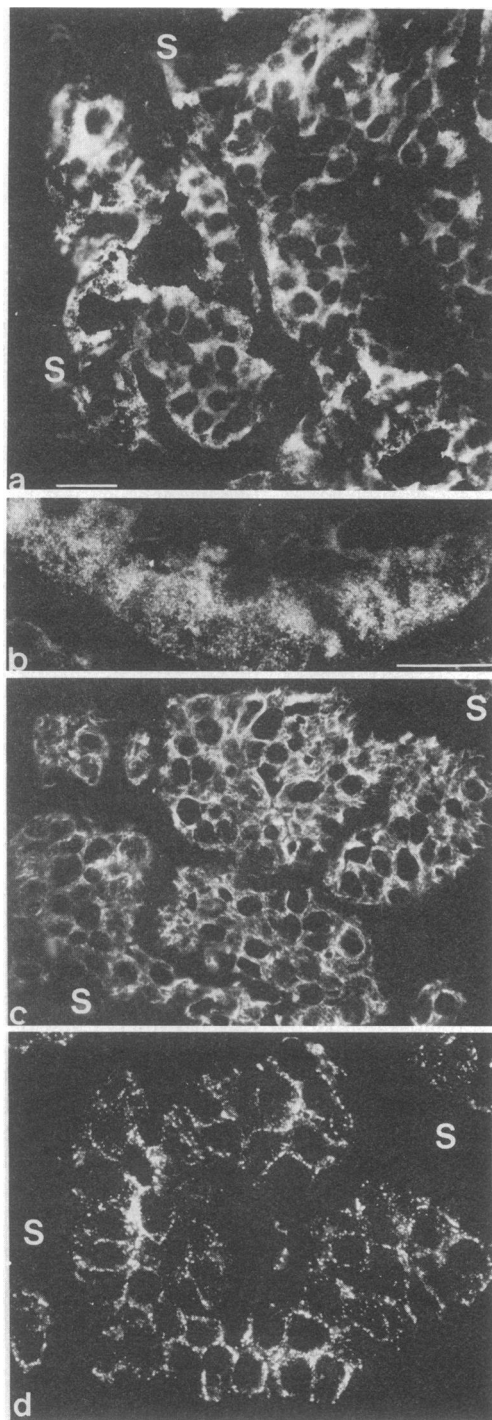


FIG. 4. Immunofluorescence microscopy on frozen sections of human carcinoid of ileum with antibodies against synaptophysin (*a* and *b*; SY38), cytokeratins (*c*; K_s 18.18), and desmoplakins (*d*; DP1&2-1.15). All antibodies stain the tumor but not the stromal (S) cells. Note the finely punctate and dispersed cytoplasmic staining with SY38 (*b*). In contrast, the desmoplakins (*d*) shows a coarser punctate pattern along cell borders. (Bars = 20 μ m.)

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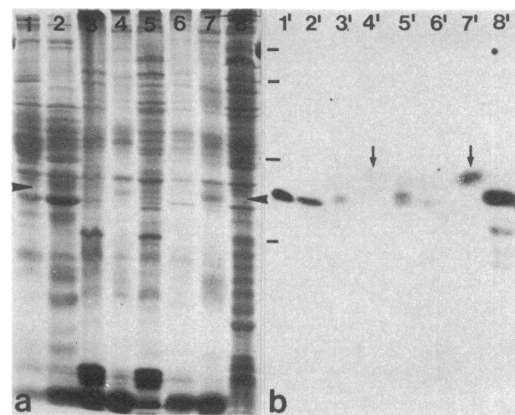


FIG. 5. Immunoblot analysis of polypeptides of subcellular fractions from normal (rat and bovine) brain and various neuroendocrine tumors, using monoclonal antibody against synaptophysin (SY38). Polypeptides separated by parallel NaDodSO₄/12.5% PAGE either were stained with Coomassie blue (*a*, lanes 1–8) or were transferred to nitrocellulose paper and treated with antibody SY38 and then with ¹²⁵I-labeled goat anti-mouse IgG antibodies (*b*, lanes 1'–8', autoradiograph). Both "low-speed supernatants" (lanes 1–5) and "high-speed pellets" (lanes 6–8), which were enriched in small vesicles, were examined. Tissues used were from rat (lanes 1 and 1') and human (lanes 2 and 2') brain or from human islet-cell carcinoma (lanes 3 and 3' and lanes 5 and 5'), paraganglioma (lanes 4 and 4' and lanes 7 and 7'), and pheochromocytoma (lanes 5 and 5' and lanes 8 and 8'). Small vertical bars in *b* denote, from top to bottom, position of reference proteins analyzed in parallel lanes (*M_r* 180,000, 96,000, 50,000, and 33,000; ref. 21). Arrowheads in *a* denote the position of brain synaptophysin (*M_r* 38,000). Note that an SY38-reactive polypeptide band is seen at a similar position in the tumor samples, with the exception of the paraganglioma samples, in which the reactive band shows a slightly lower electrophoretic mobility (arrows).

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