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Immunohistochemical Detection of Somatostatin Receptor Subtypes sst1 and sst2A in Human Somatostatin Receptor Positive Tumors

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ABSTRACT

Although *in situ* hybridization has been used to examine the distribution of messenger RNA for somatostatin receptor subtypes (sst) in human tumors, the cellular localization of sst_1 and sst_{2A} receptors has not been reported. In this study, we describe the cellular localization of human sst_1 and sst_{2A} receptor proteins in both cryostat- and paraffin-embedded sections of 25 human tumor tissues using two recently developed polyclonal antibodies. Six somatostatin (SS) receptor (SSR) positive tumors (two gastrinomas, three carcinoids, one pheochromocytoma) and one SSR negative tumor (renal cell carcinoma), selected by positive and negative SSR autoradiography, respectively, were studied by both immunohistochemistry and Western blot analysis. The six SSR positive tumors expressed sst_{2A}, while 4 of 5 expressed sst_1 as well. The SSR negative tumor did not express either sst_1 or sst_{2A}. Western blot analysis of wheat germ agglutinin purified membrane proteins confirmed the presence of the sst_1 and sst_{2A} glycosylated receptors. The paraffin-embedded sections gave best information with respect to the subcellular localization. Sst_1 immunoreactivity was observed both on the membrane and in the cytoplasm, while sst_{2A} showed predominantly membrane-associated immunoreactivity. This subcellular distribution of sst_1 or sst_{2A} receptors was confirmed in paraffin-embedded sections of 8 additional intestinal carcinoids, 5 gastrinomas and 5 pheochromocytomas. Sst_1 receptors were detected in 7 out of 8 carcinoids, in all gastrinomas, and in 4 out of 5 pheochromocytomas, while 6 out of 8 carcinoids, all gastrinomas, and 3 out of 5 pheochromocytomas expressed sst_{2A} receptors. In conclusion, sst_1 and sst_{2A} receptors show a differential subcellular localization in human SSR positive tumors. The use of SSR subtype selective antibodies to detect the subcellular distribution of SSR subtypes in individual tumor cells is an important step forward to understand more about the pathophysiological role of the different SSR subtypes in human tumors. (*J Clin Endocrinol Metab* **84:** 775–780, 1999)

A large number of human neuroendocrine tumors express somatostatin (SS) receptors (SSR) (1). Five SSR subtype genes, named sst_{1-5} have been identified and characterized (2). Two isoforms of sst_2 (sst_{2A} and sst_{2B}) are generated by alternative splicing. Until now, the detection of SSR in human tumors has been performed either by ligand binding to membrane homogenates or tissue slices to detect combined SSR binding activity, or by messenger RNA (mRNA) analysis using either *in situ* hybridization (ISH), RNAse protection, or reverse transcriptase polymerase chain reaction (RT-PCR) to detect mRNA for each receptor subtype. While the use of *in vitro* SSR autoradiography and ISH has provided significant information regarding the heterogeneity of SSR expressed in tumors (1, 3), the precise cellular localization of SSR's has been difficult to establish. Recently, two SSR subtype specific polyclonal antibodies have been developed (4, 5). The sst_1 antibody (code-named R1–201) was raised against a 15-amino acid peptide corresponding to a unique sequence in the carboxyl terminus and characterized by immunoprecipitation of photoaffinity-labeled sst_1 receptor (4). The sst_{2A} antibody (code-named R2–88) was raised against a 22-amino acid peptide located in the C-terminal region of the rat sst_{2A} receptor and characterized by immunoprecipitation of photoaffinity labeled sst_{2A} receptor as well as by immunoblot and immunohistochemistry (IHC) on cells transfected with complementary DNA encoding sst_{2A} (5, 6). The peptide sequences used to raise both antibodies are conserved in the rat, mouse, and human forms (4, 5). The sst_{2A} antibody has recently been used to detect sst_{2A} in rat brain, pancreas, and pituitary sections (6–8). In this study we have used both antibodies to characterize the subcellular localization of sst_1 and sst_{2A} in human neuroendocrine tumors.

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Materials and Methods

Tumor samples

Tumor tissue was obtained immediately after surgical removal and either frozen in liquid nitrogen through isopentane or fixed in 4% paraformaldehyde (PF) overnight. Diagnosis was made on the basis of clinical and biochemical characteristics of the patients, in combination with IHC of the tumor samples. All patients gave their informed consent for the use of tumor material for research purposes.

Immunohistochemistry

Frozen material. Five-micrometer cryostat sections were air-dried, fixed for 10 min with 10% PF, washed once with tap water, once with phos-

TABLE 1. SSR subtype sst_1 and sst_{2A} expression in human tumors as determined by SSR autoradiography, immunohistochemistry (IHC), and Western blotting

Number	Tumor type	SSR autoradiography		IHC[a]		Western blotting	
		SS-28	T3O	sst_1	sst_{2A}	sst_1	sst_{2A}
1	Gastrinoma	nt	+	+	+	nt	+
2	Gastrinoma	+	+	nt	+	+	+
3	Pheochromocytoma	+	+	±	+	−	+
4	Intestinal carcinoid	+	+	+	+	+	+
5	Intestinal carcinoid	+	+	+	+	+	+
6	Intestinal carcinoid	+	+	nt	+	−	+
7	Renal cell cancer	−	−	−	−	−	−

nt, not tested; −, negative; ±, weakly positive; +, positive. SS-28: [^{125}I-Tyr^0]SS-28, T3O: [^{125}I-Tyr^3]octreotide.
[a] IHC in cryostat- and/or paraffin-embedded sections.

phate buffered saline (PBS), and incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% BSA). Thereafter, the sections were incubated overnight at 4 C with the sst_1 (R1–201) and sst_{2A} (R2–88) antibodies in a dilution of 1:1000. Finally, a standard streptavidin-biotinylated-peroxidase complex (ABC) kit (Biogenix, San Ramon, CA) was used according to the manufacturers instructions to visualize the bound antibodies.

Paraffin-embedded material. Five-micrometer sections were deparafinized, dehydrated, exposed to microwave heating (in citric acid buffer, 10 min at 100 C), rinsed in tap water (1x) and PBS (1x), and processed further as described above for the cryostat sections ($sst_{1\ and\ 2A}$ antibody dilutions: 1:500). Negative controls for IHC included omission of the primary antibody and preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 0.3 μg/mL = 100 nM).

Western blot analyis

Membranes were prepared from human tumors or cell lines transfected to overexpress either the rat sst_{2A} receptor (GH-R2) or the rat sst_1 receptor (GH-R1) (4–6). Glycosylated proteins were purified by wheat germ agglutining (WGA) affinity chromatography as previously decribed (8). Either unpurified or WGA-purified membrane proteins were subjected to electrophoresis on 12% SDS polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes electrophoretically (4–6). After blocking, blots were incubated overnight at 4 C with 1:10,000 dilution of the appropiate antibody. Immunoblots were blocked, washed, and developed as described previously (5–8).

SSR autoradiography

SSR autoradiography was carried out as described previously (9). Ten-micrometer sections were mounted onto precleaned gelatin coated microscope slides and stored at −80 C. To wash out endogenous somatostatin, the sections were preincubated at room temperature for 10 min in 170 mM Tris-HCl pH 7.4. Thereafter, the sections were incubated for 60 min at room temperature in 170 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1% BSA, 40 μg/mL bacitracin in the presence of [^{125}I-Tyr^3]-octreotide (about 80–160 pmol/L) or [^{125}I-Tyr^0]SS28 (final concentration 80–160 pmol/L; ANAWA Laboratories, Wangen, Switzerland). Nonspecific binding was determined in a sequential section in the presence of excess unlabeled Tyr^3-octreotide (1 μM) or SS-28 (1 μM). The incubated sections were washed twice for 5 min in incubation buffer containing 0.25% BSA and once in incubation buffer without BSA. After a short wash with distilled water to remove salt, the sections were air dried and exposed to Kodak X-OMAT AR or Hyperfilm^{TM}-^{3}H (Amersham, Buckinghamshire, United Kingdom) for 3–7 days in x-ray cassettes. Histology was performed on hematoxylin-azophloxine stained sequential cryosections.

Results

Seven human neuroendocrine tumors of different origin were selected on the basis of positive and negative SSR autoradiography (Table 1). Figure 1 shows the localization of sst_1 (Fig 1, A, B, E) and sst_{2A} receptors (Fig. 1, C, D, F) in a human gastrinoma. For sst_1 receptors immunohistochemical staining was observed both in the cytoplasm and at the cell membrane. This was seen most clearly in the paraffin-embedded sections (1B). Sst_{2A} receptors were preferentially localized at the cell membrane (1D). Both intestinal carcinoids examined by IHC-expressed sst_1 and all three carcinoids tested expressed sst_{2A} (Table 1). The other gastrinoma expressed sst_{2A} as well. The localization of sst_1 receptors in the sst_1-positive carcinoid was primarily cytoplasmic as well (1G). In addition, mainly membrane-localized sst_{2A} receptors were found in the pheochromocytoma (1H). The pheochromocytoma was slightly sst_1 positive as well. Neither sst_1 nor sst_{2A} immunostaining was observed in the stromal compartment of any of the tumors, although the endothelial cells of some tumoral vessels were sst_{2A} positive (not shown). The renal cell carcinoma, which was selected on the basis of negative SSR autoradiography using [^{125}I-Tyr^0]somatostatin-28 and [^{125}I-Tyr^3]octreotide showed no positive staining by either the sst_1 and sst_{2A} antibody (Table 1).

To enrich the tumor extracts for the glycosylated receptor proteins, solubilized membrane proteins were partially purified by WGA-affinity chromatography before immunoblotting. Figure 2 compares the results of the sst_1 and sst_{2A} analysis in a gastrinoma and an intestinal carcinoid to the staining observed in an SSR negative pituitary cell line (GH12C1) transfected to express either sst_1 and sst_{2A}. In each case, the most darkly stained band shows the broad migration pattern and size range characteristic of sst receptors. Immunostaining of this band was completely inhibited by 0.5–1 μM antigen peptide (Fig. 2). Data from all tumors examined are summarized in Table 1. The apparent molecular weight (Mw) for each receptor subtype varied between tumors: the observed Mw for sst_1 ranged from 45 to 80 kDa, whereas that for sst_{2A} varied from 60 to 85 kDa. This variability is consistent with differential glycosylation occuring in individual tumors and resembles what was previously observed in tumor cell lines (4–5) and rat tissues (6–8).

To further confirm the differential subcellular localization of sst_1 and sst_{2A} receptors in the tumor cells, we performed immunohistochemical analysis of both receptor subtypes in paraffin-embedded sections of eight additional intestinal carcinoids, five gastrinomas, and five pheochromocytomas. Sst_1 receptors were detected in seven out of eight carcinoids, in all gastrinomas (n = 5), and in four out of five pheochromocytomas. Positive immunostaining for sst_{2A} receptors was found in 6 out of 8 carcinoids, in all gastrinomas (n = 5), and in three out of five pheochromocytomas. This is shown in Table 2. In all

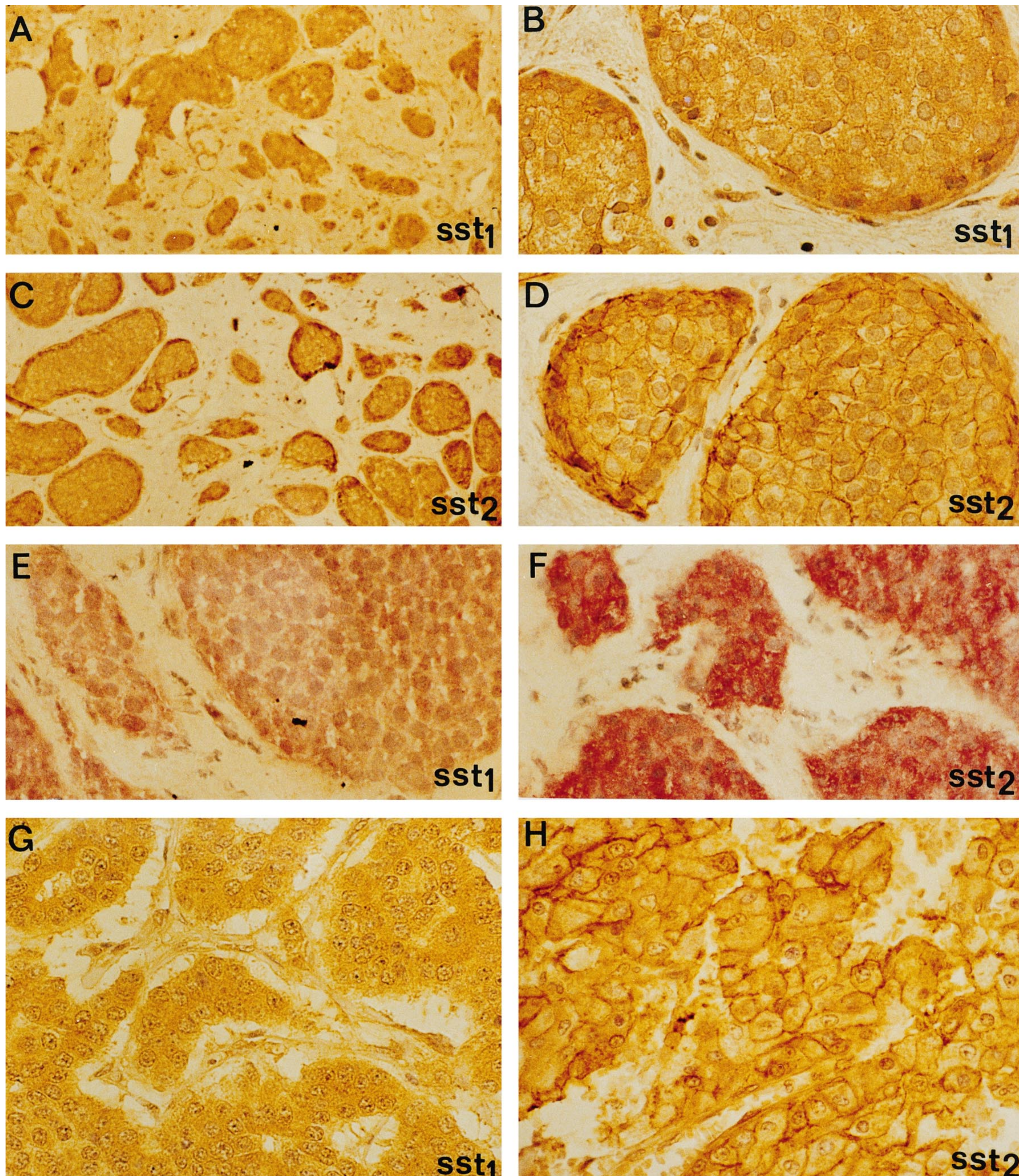

FIG. 1 Localization of sst_1 and sst_{2A} subtypes in a human SSR positive tumors. Immunohistochemical detection of sst_1 (R1–201 antibody) and sst_{2A} (R2–88 antibody) using two recently developed polyclonal antibodies (4–8) in human neuroendocrine tumors. A-D, gastrinoma, paraffin-embedded sections; E-F, gastrinoma, cryostat sections (A, B, E: sst_1, C, D, F: sst_{2A}). G; sst_1 immunoreactivity in paraffin-embedded section of a human carcinoid; H, sst_{2A} immunoreactivity in paraffin-embedded section of a human pheochromocytoma. Note the primarily cytoplasmic immunohistochemical localization of sst_1 and the preferentially membrane-associated sst_{2A} immunoreactivity.

tumors specificity of the staining with the sst_1-specific antibody (R1–201) and with the sst_{2A}-specific antibody (R2–88) was confirmed by the abolishment of immunostaining when the antibodies were pre-absorbed with 100 nM of the respective immunizing peptides. Figure 3 shows examples of the neutralization of the predominant cytoplasmic staining for sst_1-receptors in an intestinal carcinoid (Fig. 3, A, B) and in a gastrinoma (Fig. 3, C, D), as well as for the predominant mem-

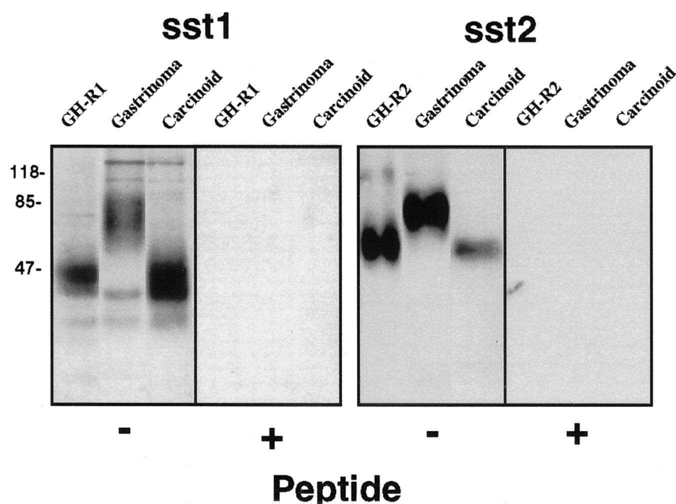

FIG. 2. Immunoblot analysis of sst_1 and sst_{2A} in human tumors. Either unpurified membrane protein (GH-R2 cells, 5 μg) or WGA-purified membrane proteins (GH-R1 cells, gastrinoma, or carcinoid, purified from 35 μg, 300 μg, and 100 μg of membrane protein, respectively) were separated on a 12% SDS polyacrylamide gel. After transfer to polyvinylidene difluoride (PVDF) membrane, the proteins were immunoblotted with 1:10,000 dilution of sst_1- (R1–201) or sst_{2A} (R2–88) antibody in the absence (−) or presence (+) of either 1 μM sst_1 peptide or 0.5 μM sst_{2A} peptide antigen. Mw markers (in kDa) are shown on the left.

TABLE 2. Immunohistochemical detection of SSR subtypes sst_1 and sst_{2A} in paraffin embedded sections of human neuroendocrine tumors

Number	Tumor type	SSR subtype	
		sst_1	sst_{2A}
8	Intestinal carcinoid	+	+
9	Intestinal carcinoid	+	−
10	Intestinal carcinoid	−	−
11	Intestinal carcinoid	+	+
12	Intestinal carcinoid	+	+
13	Intestinal carcinoid	+	+
14	Intestinal carcinoid	+	+
15	Intestinal carcinoid	+	+
16	Gastrinoma	+	+
17	Gastrinoma	+	+
18	Gastrinoma	+	+
19	Gastrinoma	+	+
20	Gastrinoma	+	+
21	Pheochromocytoma	−	−
22	Pheochromocytoma	+	+
23	Pheochromocytoma	+	+
24	Pheochromocytoma	+	−
25	Pheochromocytoma	+	+

For immunohistochemical detection, two recently developed sst_1 (R1-201) and sst_{2A} (R2-88)-specific polyclonal antibodies were used (4–6)

brane-associated staining of sst_{2A}-receptors in a gastrinoma (Fig. 3, E, F) and a pheochromocytoma (Fig. 3, G, H). In the tumors studied, irrespective of the tumor type, we confirmed the primarily cytoplasmic localization of sst_1 receptors and the preferential localization of sst_{2A} receptors at the cell membrane.

Discussion

Along with the widespread actions of SS, many SS-target tissues as well as many neuroendocrine tumors often originating in these target tissues, have been shown to express SSR's (1, 10). Until now SSR detection has been performed using ligand binding studies on membrane preparations or on frozen sections. In addition, the recent cloning and characterization of the genes of five SSR subtypes (2) has provided molecular biological tools, *i.e.* RT-PCR and ISH techniques, to study SSR subtype expression in normal and tumoral tissues (3). Moreover, the development of SSR subtype specific antibodies (4–8) now allows a detailed study of the cell types and cellular localization of SSR subtype proteins. In the present study we describe the cellular localization of sst_1 and sst_{2A} receptors in human neuroendocrine tumors of different origin. Specificity of the sst_1 and sst_{2A} antibodies was confirmed by immunohistochemical identification of approximately 60 and 85 kDa receptor proteins, respectively, in tumors by Western blotting experiments. The high frequency of expression of sst_1 and sst_{2A} receptor proteins in gastrinomas and carcinoids is in agreement with a predominant expression of sst_1 and sst_{2A} mRNAs in these tumor types (11).

The presence and possible functional significance of SSR's and SSR subtypes expressed in normal and tumoral neuroendocrine tissues has been reviewed (2, 10, 12). While sst_{2A} and sst_5 are involved in the inhibition of hormone secretion, the functional role of the other SSR subtypes is less clear. In cells transfected with SSR subtype genes, agonist activation of sst_1, sst_{2A}, and sst_5 seems involved in the antiproliferative action of SS(-analogs) via distinct as well as overlapping mechanisms (2). Whether such mechanisms also occur in human tumor cells expressing these particular subtypes remains to be established.

In the present study we found immunoreactive sst_1 primarily in the cytoplasm, whereas sst_{2A} was predominantly expressed at the cell membrane, irrespective of the tumor type. The membrane-localization of sst_2 is in agreement with a recent study by Janson *et al.* (13) in human carcinoid tumors. The precise functional significance of our observations is unclear. The cytoplasmic sst_1 may represent either neosynthesized or internalized receptors, as suggested previously to explain the cytoplasmic localization of sst_{2A} in specific rat brain neurons in regions with high somatostatin expression (14). However, previous studies with COS or CHO cells transfected with individual SSR subtypes have shown that sst_1 is internalized more poorly than sst_{2A} after hormone binding (15, 16). If endogenous receptors in tumors behaved similarly, one would predict that more of the sst_{2A} than the sst_1 receptor would be internalized in tumors expressing both receptor subtypes. Instead we found the inverse distribution. Moreover, agonist exposure upregulates sst_1 expression in CHO-K1 cells expressing this SSR subtype (16), while chronic SS exposure of GH_4C_1 pituitary cells increases SSR numbers (17). This increase in SSR number in GH_4C_1 cells was independent of new protein synthesis, and a potential mechanism could be changes in the intracellular distribution of SSRs (17). In this respect our observation of a predominant cytoplasmic localization of sst_1 receptors in human tumor cells may also reflect a capacity of the tumor cells expressing this SSR subtype to increase SSR numbers after agonist exposure. This suggests that treatment of sst_1 receptor-expressing cells with sst_1-selective agonists may not

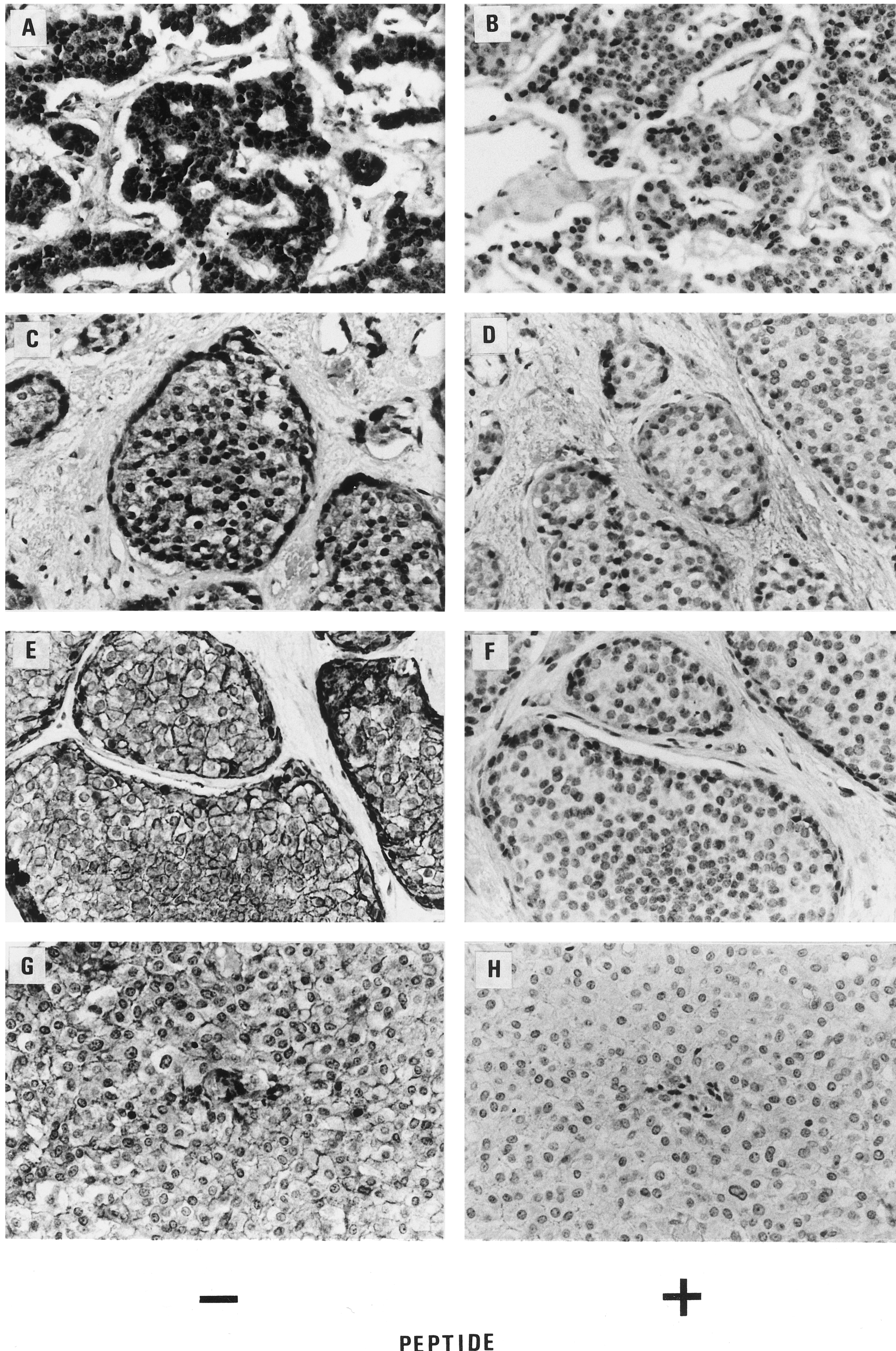

FIG. 3. Displacement of immunohistochemical staining of sst1 (R1–201 antibody) and sst_{2A} (R2–88 antibody) subtypes in human SSR positive tumors by preabsorbtion of the antibodies with 100 nM of the respective immunizing peptides. A and B, intestinal carcinoid (sst_1); C and D, gastrinoma (sst_1); E and F, gastrinoma (sst_{2A}); G and H, pheochromocytoma (sst_{2A}).

result in a desensitization to treatment as has been observed in the majority of patients with islet-cell tumors and carcinoids treated with sst_2-selective octapeptide SS-analogs (18). Clearly additional studies will be needed to establish whether this differential localization of sst_1 and sst_{2A} is a general phenomenon and to determine the biochemical mechanisms responsible.

In summary, the use of SSR subtype specific antibodies now allows a detailed examination of the subcellular localization of SSR subtypes in individual cells. This is an important step toward understanding more about the functional role of the individual SSR subtypes in human SSR positive tumors.

References

1. **Reubi JC, Laissue J, Krenning E, Lamberts SWJ.** 1992 Somatostatin receptors in human cancer: incidence, characteristics, functional correlates and clinical implications. J Steroid Biochem Mol Biol. 43:27–35.
2. **Reisine T, Bell GI.** 1995 Molecular biology of somatostatin receptors. Endocr Rev. 16:427–442.
3. **Reubi JC, Schaer JC, Waser B, Mengod G.** 1994 Expression and localization of somatostatin receptor SSTR1, SSTR2, and SSTR3 messenger RNAs in primary human tumors using *in situ* hybridization. Cancer Res. 54:3455–3459.
4. **Gu Y-Z, Brown PJ, Loose-Mitchell DS, Stork PJS, Schonbrunn A.** 1995 Development and use of a receptor antibody to characterize the interaction between somatostatin receptor subtype 1 and G proteins. Mol Pharmacol. 48:1004–1014.
5. **Gu Y-Z, Schonbrunn A.** 1997 Coupling specificity between somatostatin receptor sst2A and G proteins: isolation of the receptor-G protein complex with a receptor antibody. Mol Endocrinol. 11:527–537.
6. **Dournaud P, Gu YZ, Schonbrunn A, Mazella J, Tannenbaum GS, Beaudet A.** 1996 Localization of the somatostatin receptor sst2A in rat brain using a specific anti-peptide antibody. J Neurosci. 16:4468–4478.
7. **Mezey E, Hunyady B, Mitra S, et al.** 1998 Cell specific expression of the sst2A and sst5 somatostatin receptors in the rat anterior pituitary. Endocrinology. 139:414–419.
8. **Hunyady B, Hipkin RW, Schonbrunn A, Mezey E.** 1997 Immunohistochemical localization of somatostatin receptor sst2A in the rat pancreas. Endocrinology. 138:2632–2635.
9. **Hofland LJ, de Herder, WW, Visser-Wisselaar HA, et al.** 1997 Dissociation between the effects of somatostatin (SS) and octapeptide SS-analogs on hormone release in a small subgroup of pituitary and islet cell tumors. J Clin Endocrinol Metab. 82:3011–3018.
10. **Lamberts SWJ, Krenning EP, Reubi JC.** 1991 The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr Rev. 12:450–482.
11. **Schaer J-C, Waser B, Mengod G, Reubi JC.** 1997 Somatostatin receptor subtypes sst_1, sst_2, sst_3 and sst_5 expression in human pituitary, gastroentero-pancreatic and mammary tumors: comparison of mRNA analysis with receptor autoradiography. Int J Cancer. 70:530–537.
12. **Hofland LJ, Visser-Wisselaar HA, Lamberts SWJ.** 1995 Somatostatin analogs: clinical application in relation to human human somatostatin receptor subtypes. Biochem Pharmacol. 50:287–297.
13. **Janson ET, Stridsberg M, Gobl A, Westlin J-E, Oberg K.** 1998 Determination of somatostatin receptor subtype 2 in carcinoid tumors by immunohistochemical investigation with somatostatin receptor subtype 2 antibodies. Cancer Res. 58:2375–2378.
14. **Dournaud P, Boudin H, Schonbrunn A, Tannenbaum GS, Beaudet A.** 1998 Interrelationships between somatostatin sst2A receptors and somatostatin containing axons in rat brain: evidence for regulation of cell surface receptors by endogenous somatostatin. J Neurosci. 18:1056–1071.
15. **Nouel D, Gaudriault G, Houle M, et al.** 1997 Differential internalization of somatostatin in COS-7 cells transfected with sst_1 and sst_2 receptor subtypes: a confocal microscopic study using novel fluorescent somatostatin derivatives. Endocrinology. 138:296–306.
16. **Hucovic N, Panetta R, Kumar U, Patel YC.** 1996 Agonist-dependent regulation of cloned human somatostatin receptor types 1–5 (hSSTR1–5): subtype selective internalization or upregulation. Endocrinology. 137:4046–4049.
17. **Presky DH, Schonbrunn A.** 1988 Somatostatin pretreatment increases the number of somatostatin receptors in GH_4C_1 pituitary cells and does not reduce cellular responsiveness to somatostatin. J Biol Chem. 263:714–721.
18. **Lamberts SWJ, van der Lely AJ, de Herder WW, Hofland LJ.** 1996 Octreotide. N Engl J Med. 334:246–254.