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MULTIPLE GENE TRANSCRIPTS OF THE SOMATOSTATIN RECEPTOR SSTR2: TISSUE SELECTIVE DISTRIBUTION AND cAMP REGULATION[1]

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The rodent SSTR2 mRNA has been reported to be alternatively spliced to generate long (SSTR2A) and short (SSTR2B) receptor isoforms which differ in sequence at their C-terminal regulatory domains. By extending the 3' nucleotide sequence of the human gene (hSSTR2) we show highly conserved intron/exon boundaries suggesting that hSSTR2 is also capable of generating spliced variants. mRNA blots of rat tissues reveal 2 transcripts of 2.8 and 2.3 kb that are differentially expressed in brain regions and multiple peripheral organs. The 2.3 kb mRNA is preferentially expressed in pituitary tumor cells (AtT-20 mouse, GH_3 rat, human prolactinoma, human somatotroph adenoma), but not in rat or human insulinoma cells. This transcript shows 4 fold induction by forskolin in AtT-20 cells suggesting cAMP dependent control of SSTR2 gene expression. The abundant expression of SSTR2 gene, the occurrence of 2 isoforms and evidence of extensive regulation at both gene and peptide levels, suggests that SSTR2 is the principal SST-14 selective subtype.

INTRODUCTION - Somatostatin (SST) consists of 2 potent neuropeptides, SST-14 and SST-28, which exert a multitude of effects on a wide range of tissue targets via G protein coupled membrane receptors (1). Recently, five SSTR genes have been cloned and shown to consist of a family of genes encoding for distinct receptor subtypes (2-9). SSTR1,2,3, and 4 are closely related in size, structure, and selectivity for SST-14 binding (2-6, 8,9) whereas a recently cloned fifth SSTR exhibits preferential agonism for SST-28 (7). The human genes for SSTR1,2,3, and 4 have been structurally characterized, localized on separate chromosomes (14, 17, 22, 20 for hSSTR1,2,3, and 4 respectively) and reported to be intronless (2,8,9). Recent studies, however, suggest that the mouse SSTR2 mRNA is capable of generating two different receptor forms - a long (SSTR2A), and a short (SSTR2B) variant, through alternate mRNA splicing (10). Preliminary data indicate that rat SSTR2 mRNA also generates the 2 spliced mRNA variants corresponding to mouse SSTR2A and B, but whether the same applies to human SSTR2 is unknown (10). In the present study, we have characterized by genomic sequencing the 3' end of human SSTR2 DNA to determine whether a splice site exists in this gene homologous to that in the rodent. Second, we have characterized the

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relative level of expression of mRNA transcripts for SSTR2A and SSTR2B in normal rat tissues and compared this pattern with that in rat and mouse pituitary and islet tumor cell lines. Third, we have investigated SSTR2 gene expression in human pituitary and islet tumors by RNA blot analysis. Finally, we have elucidated SSTR2 gene regulation by activators of protein kinase A, protein kinase C, and NMDA receptor agonists.

MATERIALS AND METHODS

Normal Tissues - Tissues were obtained by gross dissection from 150 g male C.D. rats, frozen in liquid nitrogen, and stored at -80°C.

Tumor Cells - AtT-20-D16V mouse pituitary cells (11), GH_3 rat pituitary cells (11), RINm5f rat insulinoma cells (12), 1027 B_2 rat islet somatostatinoma cells (13), PC12 rat pheochromocytoma cells (12), and COS-7 monkey kidney cells (12) were obtained and cultured as previously described. Cells were grown as monolayers in 10 cm diameter plastic Petri dishes and used at 7-14 days at ~ 70% confluency. For SSTR2 gene regulation experiments groups of 5 culture dishes containing AtT-20 cells were incubated for 16 h with control medium (Dulbecco's Modified Eagle's Medium, 5% fetal bovine serum supplemented with Ser-X-tend) or medium containing test agents [forskolin 10 μM, phorbol 12-myristate 13-acetate, (PMA) 0.4 μM, and quinolinic acid 5 mM]. At the end of incubation, cells were harvested for RNA extraction.

Human Pituitary and Islet Cell Tumors - Pituitary tumor tissue was obtained at surgery from 3 patients, one with a nonfunctioning adenoma, one with a prolactinoma, and one with a somatotroph adenoma. The diagnosis in each case was established on the basis of clinical and hormonal data and confirmed by immunocytochemistry of the removed tumor tissue. A fourth tumor consisted of a benign insulinoma diagnosed clinically and histologically. Tumor tissue in each instance was collected in liquid nitrogen and kept at -80°C.

RNA Extraction and Northern Blot Analyses - Total RNA was isolated by the guanidinium-isothiocyanate acid phenolchloroform extraction method (14). 20-40 μg of RNA was denatured in formamide and formaldehyde and fractionated on a 1.5% agarose/formaldehyde gel. The RNA was then transferred to Nytran nylon membrane and hybridized in 50% formamide at 60°C to [α-^{32}P] UTP labeled cRNA probe or at 42°C to [α-^{32}P] d-CTP labeled DNA probe. Blots were washed at moderate stringency and exposed to Kodak XAR-5 film at -80°C for 1-3 weeks with intensifying screens.

Probes - Full length human SSTR2 cDNA was synthesized by polymerase chain reaction (PCR) using synthetic oligonucleotide primers to the 5' and 3' termini of SSTR2. The product was cloned into p Bluescript from which cRNA probes were generated using T7 RNA polymerase.

Sequencing of the 3' End of hSSTR2 - A 15 kb *Sac* I human genomic fragment cloned in SP73 and encoding the human SSTR2 gene was obtained from L. Demchyshyn, Toronto (8,9). From this a 3 kb genomic fragment was subcloned into p Bluescript using a *Hind III* site that is present in the 15 kb genomic fragment. Sequence analysis was performed using the dideoxy chain termination method (15) with a Pharmacia T7 sequencing kit and showed that the *Hind III* site is located 0.5 kb downstream of the SSTR2 stop codon described in Yamada et al (2).

RESULTS

Sequence Analysis of the 3' End of hSSTR2 - Analysis of the available nucleotide sequence of the hSSTR2 gene (2) showed that the 5' end of the putative intron is conserved in the human. However, the nucleotide sequence of the 3' end of this intron as well as the sequence of the exon has not been reported. Accordingly, we extended the nucleotide sequence analysis of the 3' end of the hSSTR2 gene (Fig. 1) and determined that the 3' end of the putative intron is located 224 b.p. downstream of the first hSSTR2 stop condon (2). In addition to the conserved intron/exon

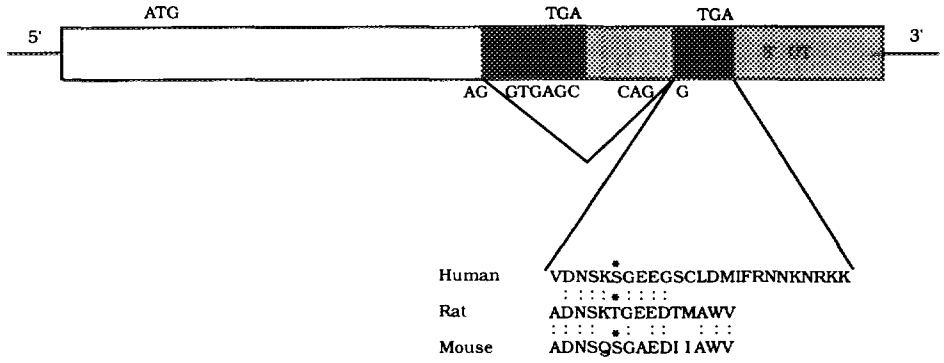

FIGURE 1. Schematic representation of the human SSTR2 gene showing the 2 spliced variants. The box represents the unspliced transcript. The predicted splice junctions, used to generate the smaller transcript, are shown by a triangle. The black boxes represent the coding sequences of the variable C-terminal regions of the 2 transcripts. The predicted C-terminal amino acid sequence of the hSSTR2 spliced variant (or hSSTR2B) is aligned with the rat and mouse sequences (2,10). The shaded areas represent transcribed but untranslated DNA. The predicted translational start (ATG) and stop (TGA) codons are illustrated. Also depicted is the sequence of the 5' and 3' end cryptic splice sites determined from the hSSTR2 gene. * denotes putative phosphorylation site.

boundaries surrounding the alternatively spliced exon in both rodent and human SSTR2 genes, the amino acid sequences of the predicted exons display ~ 60% identity. A comparison of the C-terminal domain of human, mouse, and rat SSTR2A shows 3 putative Ser/Thr phosphorylation sites in each receptor (2,3,10). In contrast the homologous region of human, rat and mouse SSTR2B displays only one such site (Fig. 1).

SSTR2 Gene Expression in Normal Rat Tissues - Northern blot analysis of total RNA from normal rat tissues showed 2 transcripts of 2.8 kb and 2.3 kb in colon, kidney, cerebral cortex, hypothalamus, anterior and posterior pituitary, and adrenal cortex but not in lung, testis, or muscle (Fig. 2). High levels of expression of this receptor gene occurred in anterior and posterior pituitary, adrenals, colon, cerebral cortex, and hypothalamus. Antrum and pancreas showed weak but definite hybridization signals. All positive tissues exhibited both transcripts but with tissue specific preferential expression of one or the other form. For instance, the 2.3 kb transcript was the dominant form in hypothalamus whereas the 2.8 kb form predominated in pituitary, colon, and adrenal.

SSTR2 Gene Expression in Cultured Mouse and Rat Tumor Cells - RNA blots of various tumor cell lines (Fig. 3) revealed a single 2.3 kb mRNA species in AtT-20 and GH_3 pituitary cell lines. RINm5f cells (known to be SSTR positive) (16), and the closely related 1027 B_2 cells, were both negative for SSTR2 mRNA. Likewise PC12 and COS-7 cells were also SSTR2 negative.

SSTR2 Gene Expression in Human Pituitary and Islet Tumors - Analysis of 3 human pituitary tumors revealed SSTR2 gene expression in the prolactinoma and somatotroph adenoma but not in the chromophobe adenoma (Fig. 4). The prolactinoma displayed high level expression of the 2.3 kb mRNA transcript and additionally small amounts of a 8.0 kb mRNA species. The somatotroph adenoma expressed only the 2.3 kb mRNA. In contrast to the pituitary tumors, the human

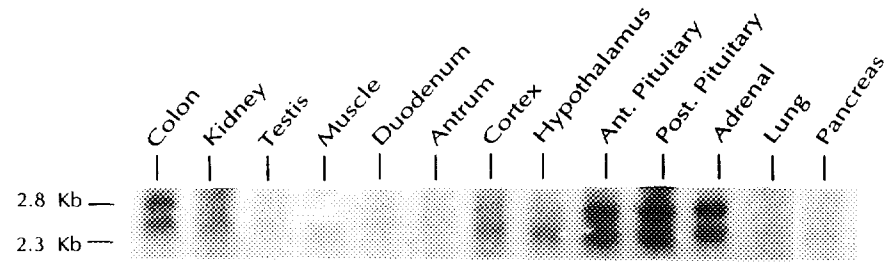

FIGURE 2. RNA blots of 40 µg total RNA from normal rat tissues hybridized to SSTR2 cRNA probe. Two transcripts of 2.8 kb and 2.3 kb are detected in colon, kidney, cerebral cortex, hypothalamus, pituitary, and adrenal cortex but none in lung, testis, or muscle. Antrum and pancreas show weak but definite hybridization signals. Note the preferential expression of the 2.3 kb transcript in hypothalamus and of the 2.8 kb form in pituitary, colon, and adrenal.

insulinoma was negative for SSTR2 gene expression. By RNA blot analysis, none of the human tumors examined displayed a 2.8 kb transcript corresponding to rat SSTR2A mRNA.

Regulation of SSTR2 Gene Expression - Treatment of AtT-20 cells with forskolin for 16 h led to a 4-fold induction in 2.3 kb SSTR2 mRNA suggesting cAMP dependent regulation of this gene (Fig. 5). Under the same conditions, PMA and quinolinic acid showed no effect. The 2.8 kb SSTR2 mRNA which is expressed at low levels in this cell (10) could not be visualized on RNA blots even after forskolin induction.

DISCUSSION - The recent molecular cloning of the SSTR has already revealed five separate genes encoding for distinct subtypes of this receptor family (2-9). In addition to this genetic diversity, the rodent form of SSTR2 features two separate isoforms SSTR2A and SSTR2B encoded by two separate mRNA transcripts of 2.6 and 2.3 kb length (10). Sequence analysis of cDNAs from rat and mouse cells suggests that the smaller transcript is a spliced product of the larger form. The 5' donor site of the alternatively spliced intron of the SSTR2 gene is not readily apparent since it is located within the coding sequence (Fig. 1). In the 2.3 kb spliced transcript, the C-terminal 38 codons of the unspliced 2.6 kb transcript are replaced by a sequence encoding 15 different codons. Therefore, the amino acid sequence of the polypeptides predicted from the nucleotide sequence of both transcripts differ only in length (38 vs 15 amino acid residues) and in the composition of the C-terminal tail. Sequence analysis of this region of the human SSTR2 gene in the present study shows that the exon/intron boundaries, as well as the amino acid sequence of the putative second exon

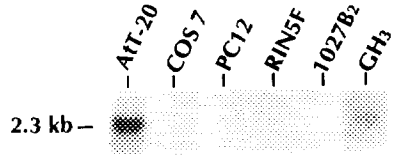

FIGURE 3. RNA blot analysis of 20 µg total RNA from mouse and rat tumor cells hybridized to SSTR2 cRNA probe. A single 2.3 kb mRNA species is detected in the 2 pituitary cells (AtT-20, GH_3), but not in islet cells (RIN, 1027 B2) or PC12 and COS-7 cells.

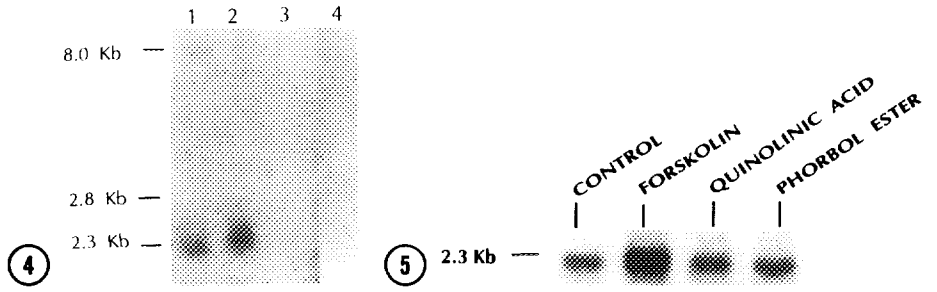

FIGURE 4. RNA blots of human pituitary and islet tumors. 20 µg total RNA was hybridized with SSTR2 cRNA probe. Lane 1 (prolactinoma) shows 2 bands of 2.3 kb and 8.0 kb. Lane 2 (somatotroph adenoma) displays a single 2.3 kb band. Lanes 3 (chromophobe adenoma) and 4 (insulinoma) are both negative.

FIGURE 5. RNA blots of AtT-20 cells exposed for 16 h with forskolin, the phorbol ester PMA, and the NMDA receptor agonist quinolinic acid. 20 µg total RNA was hybridized to SSTR2 cDNA probe. Forskolin induced a 4-fold increase in SSTR2 mRNA whereas PMA and quinolinic acid were without effect.

are highly conserved between the murine and human genes, suggesting that human SSTR2 is also capable of generating spliced variants.

By Northern blot analysis, rat SSTR2 gene expression has been previously reported in a limited study in which the only two positive tissues, brain and pituitary, expressed equal abundance of the short and long mRNA transcripts (3). Here we have surveyed the expression of SSTR2 mRNA in a large number of putative receptor positive and receptor negative rat tissues and show an abundant expression of this gene in different brain regions as well as in multiple peripheral organs. mRNA for the receptor was expressed at high levels in both anterior and posterior pituitary, hypothalamus, cerebral cortex, adrenal cortex, and colon and in low amounts in the gastric antrum and pancreas. Every rat tissue that expressed SSTR2 mRNA featured 2 transcripts corresponding to the SSTR2A and B isoforms. Furthermore, the relative proportions of the two mRNA species varied between tissues. This is in contrast to mouse SSTR2 mRNA which by Northern blots of normal tissues and AtT-20 cells, consists of a single 2.3 kb mRNA transcript suggesting that the short SSTR2B isoform predominates in this tissue; the longer SSTR2B mRNA can only be detected by more sensitive techniques such as PCR (10). In human tissues, SSTR2 mRNA has been described as a 2.5 kb mRNA transcript in all receptor positive tissues, along with an 8 kb mRNA species found in occasional tissues (2). Our analysis of human tumors in the present study revealed a 2.3 kb transcript corresponding to SSTR2B in both receptor positive pituitary tumors, one of which also expressed the 8 kb form. This transcript is considerably larger in size than the 2.8 kb SSTR2A mRNA, and its presence in both normal and neoplastic human tissues suggests that it may be another receptor variant. Our finding of SSTR2 mRNA expression in GH_3 cells is consistent with the pharmacological properties of membrane receptors on these cells which display SST-14 selectivity. The solitary human somatotroph adenoma examined in this study also expressed SSTR2 mRNA. Whether these tumors will also feature other SST-14 selective

receptor subtypes, e.g. SSTR1, 3, and 4 remains to be determined. The occurrence of SSTR2 mRNA in a prolactinoma is in keeping with earlier data showing SSTR binding sites in the majority of these tumors (17). Likewise, the absence of SSTR2 mRNA in both the human and rat insulinoma cells examined in this study is consistent with earlier evidence for expression of SST-28 selective receptors on these cells (18).

G protein linked receptors undergo extensive regulation at the membrane through changes in phosphorylation (19). In the case of SSTR2 the production of 2 isoforms which differ only in their C-terminal domain, a region shown to be important for phosphorylation-dependent receptor desensitization suggests that the two variants may be differentially regulated. Besides control at the receptor protein level, however, our finding of forskolin induction of SSTR2B mRNA in AtT-20 cells suggests additional regulation of this receptor at the gene level. Whether this occurs transcriptionally through a cAMP responsive enhancer element on the gene, or through a posttranscriptional mechanism remains to be determined (20). Likewise, the question of whether the larger transcript is coregulated via the cAMP signalling pathway requires further investigation using tissues, e.g. rat pituitary, which express high levels of both isoforms.

In conclusion, our studies reveal abundant expression of SSTR2 gene in different brain regions as well as in multiple peripheral organs. This expression appears to be more widespread than that reported for the 3 other SST-14 selective receptor subtypes (2, 4-6). Furthermore, SSTR2 occurs as 2 isoforms and shows evidence of extensive regulation at both gene and protein levels, suggesting that it is the principal SST-14 selective subtype.

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