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Molecular cloning and functional expression of a brain-specific somatostatin receptor

(G protein-coupled receptor/somatostatin 14/somatostatin 28/gene)

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ABSTRACT **The PCR and conventional library screening were used to clone the brain-specific somatostatin receptor rSSTR-4 from a rat genomic library. The deduced amino acid sequence encodes a protein of 384 amino acids and displays structural and sequence homologies with members of the G protein-receptor superfamily. The amino acid sequence of rSSTR-4 is 60% and 48% identical to that of somatostatin receptors SSTR-1 and SSTR-2, respectively, two recently cloned subtypes. Competition curve analysis of the binding properties of the receptor transiently expressed in COS-1 cells revealed a higher apparent affinity for somatostatin 14 than for somatostatin 28. In contrast, the somatostatin analogs SMS 201-995, IM 4-28, and MK-678 failed to displace specific binding in transfected cells. These characteristics resemble the pharmacological binding properties of the previously described brain-specific somatostatin-receptor subtype. Examination of the tissue distribution of mRNA for rSSTR-4 revealed expression limited to various brain regions with highest levels in the cortex and hippocampus. Thus, based on the pharmacology and tissue localization of this receptor, we conclude that rSSTR-4 represents a brain-specific somatostatin receptor.**

Somatostatin 14 (SS-14), a cyclic tetradecapeptide, was originally isolated from ovine hypothalamus and found to have potent inhibitory effects on growth hormone and thyroid-stimulating hormone secretion from the anterior pituitary (1, 2). SS-14 was subsequently found to have a widespread distribution, occurring primarily in the central nervous system and gut, where it exerts inhibitory effects on secretion, both exocrine and endocrine, as well as on neural function (3). As with many small peptides, SS-14 is synthesized as part of a larger precursor molecule, from which the mature hormone is proteolytically processed. In mammals, a single gene encodes the 92-amino acid common precursor to both SS-14 and somatostatin 28 (SS-28), a second biologically active NH_2-terminally extended form of the tetradecapeptide (4).

The inhibitory actions of SS-14 and SS-28 are initiated after binding to high-affinity receptors that have been identified on target tissues (5–9). SS-14 and SS-28 appear to exert their inhibitory effects by a dual mechanism involving receptor activation of G_i, the inhibitory guanine nucleotide-binding protein (10). In part, their effects are mediated through inhibition of stimulated adenylyl cyclase activity and reduction of cAMP production (11, 12). In addition, both peptides cause membrane hyperpolarization through effects on a voltage-dependent plasma-membrane potassium channel, leading to a decrease in Ca^{2+} influx and a reduction of cytosolic Ca^{2+} concentration (13).

Based on differences in both biological and pharmacological characteristics of SS-14 and SS-28, a number of structure–activity studies have indicated the existence of multiple somatostatin receptors (14–17). The recent cloning of two somatostatin receptors, SSTR-1 and SSTR-2, from human and mouse genomic and rat cDNA libraries has confirmed that the biological effects of somatostatin are mediated by a family of receptors that are expressed in a tissue-specific manner (18, 19). Although these receptors share only 46% amino acid-sequence identity, they display similar binding affinities for SS-14 and SS-28. Because molecular cloning and Southern blotting data indicate that additional members of the somatostatin-receptor gene family exist, we used a combination of the PCR and conventional library-screening methodology to clone[§] another somatostatin-receptor subtype from a rat genomic library that has a tissue distribution limited to specific regions within the central nervous system.

MATERIALS AND METHODS

PCR Methodology and Genomic Library Screening. Partial SSTR-1 and SSTR-2 DNAs were generated from rat genomic DNA by PCR under standard conditions (20) using sequence-specific oligonucleotide primers (18). The primers were synthesized on an Applied Biosystems 391 DNA synthesizer with nucleotides encoding *Not* I restriction sites at the 5′ ends. The 700-base-pair (bp) DNA fragments of rat SSTR-1 and SSTR-2 generated by PCR were cloned into pBluescript II KS(+) (Stratagene), and the nucleotide sequence of both clones was confirmed by using the methodology outlined below.

A rat genomic library (7.5×10^5 recombinants) in bacteriophage λEMBL3 (21) was screened with SSTR-1 and SSTR-2 700-bp fragments labeled with ^{32}P by the method of random priming (22). Duplicate nylon filters were hybridized in 25% (vol/vol) formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.5% SDS/0.04% Ficoll/0.04% poly(vinylpyrrolidone)/0.04% bovine serum albumin/100 μg of sonicated and denatured salmon sperm DNA per ml at 37°C for 40 hr. Filters were washed in 1× SSPE/0.1% SDS at 50°C and exposed at −70°C with Kodak X-Omat film with enhancing screens. Phage plaques that hybridized positively on replicate filters were identified and plaque-purified by secondary and tertiary screening procedures as described (22). Of 20 clones isolated, clone Yx3, containing a 15-kilobase (kb) insert, was chosen for further analysis. Minimal-sized fragments of clone Yx3 were generated by restriction digestion, isolated, subcloned into

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Abbreviations: SS-14 and SS-28, somatostatin 14 and somatostatin 28, respectively; SSTR-1, SSTR-2, and SSTR-4, somatostatin receptor 1, 2, and 4, respectively—prefixes r and m denote rat and mouse, respectively.

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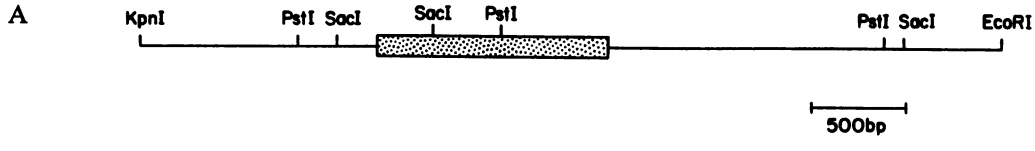
§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96544).

pBluescript II KS(+), and sequenced by using T3 and T7 primers. A 2.5-kb *Sac* I fragment contained a partial open reading frame showing strong sequence homology to SSTR-1 and SSTR-2, suggesting clone Yx3 was another somatostatin-receptor subtype, herein referred to as rSSTR-4. The sequence of the entire coding region of the putative somatostatin receptor was determined, as outlined below, by using a 4.5-kb *Kpn* I–*Eco*RI fragment as template (see Fig. 1*A*). The predicted coding region of rSSTR-4 was generated by PCR from the 4.5-kb *Kpn* I–*Eco*RI fragment and oligonucleotides corresponding to the coding region 5′ [5′-TGT GAG TTC TAA GCT TCC ACC ATG AAC ACG CCT GCA ACT CTG-3′; with the 5′ end modified to an optimal Kozak sequence (23)] and 3′ (antisense, 5′-TGT GAG TTC TGC GGC CGC TCA GAA AGT AGT GGT CTT GGT GAA AGG-3′) ends as primers. The 1.2-kb fragment generated by PCR and the above-mentioned primers contained a *Hin*dIII restriction site at the 5′ end and a *Not* I restriction site at the 3′ end. This fragment was isolated, digested with restriction enzymes, and ligated into pBluescript II KS(+) (pSSTR4/BS). Sequence of the amplified product was confirmed as outlined below.

DNA Sequencing. Genomic DNA and PCR fragments were subcloned into pBluescript II KS(+) and sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* (24) using deoxyadenosine 5-[γ-[^{35}S]thio]triphosphate and Sequenase version 2.0 (United States Biochemical). Sequencing was initiated with primers complementary to the T3 and T7 promoters of pBluescript. Subsequently, primers were synthesized as needed on the basis of newly determined sequences. Sequence analysis was done by using the Genetics Computer Group software program (25).

Expression Studies. COS-1DM cells, a subclone of COS-1 cells (provided by Jonathan Whittaker and Dennis C. Mynarcik, State University of New York at Stony Brook), were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, DC)/10% fetal calf serum/nonessential amino acids/2 mM glutamine/1 mM pyruvate/gentamycin at 500 μg/ml in 5% CO_2 at 37°C. The 1.2-kb PCR fragment was directionally inserted into the eukaryotic expression vector pCDNA1 (Invitrogen, San Diego), a modified form of expression vector pCDM8 (26). Transfection of the resulting construct into COS-1DM cells grown in 100-mm-diameter plates was accomplished by using the calcium phosphate-transfection protocol (27). After removal of the transfection medium, the cells were rinsed twice with growth medium, replaced with fresh growth medium, and incubated for 4 hr at 37°C under 5% CO_2, at which time they were pooled and then aliquoted into 24-well 16-mm diameter plates.

Binding studies were done by using intact cells 48 hr after transfection. Transfected cells were washed with 1.0 ml of Dulbecco's phosphate-buffered saline and then with 1.0 ml of DMEM containing 50 mM Hepes, pH 7.4, aprotinin at 200 kallikrein international units/ml, 0.1% bacitracin, and 0.1% bovine serum albumin (incubation buffer). Cells were incubated at room temperature in triplicate with 0.25 ml of incubation buffer containing 0.03 nM ^{125}I-labeled [Tyr^{11}]SS-14 (specific activity, 2000 Ci/mmol, Amersham; 1 Ci = 37 GBq) with or without various concentrations of unlabeled SS-14, SS-28, [D-Trp^{8}]SS-14 (Bachem), SMS 201-995 (Sandoz Pharmaceutical), IM 4-28 (ref. 16; from D. Coy, Tulane University School of Medicine), or MK-678 (ref. 17; Merck) for 1 hr. Incubated cells were washed twice with phosphate-buffered saline and then dissolved in 0.25 ml of 0.1 M NaOH.



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   1  AAGTGAGAGAGCTGGGCGACTGGAGCTCCCGCGGACGAGGAGCGAGCGGCAGAGCAGGGATTGGCGGGTGCTCCCCGGCCC  81
  82  CCGCGCACTTCTTAGGCGGAGCGGCTGAGCGGAGCGTGTGCGCACGACCCCAGCCCGCCTCTTGGTCCTTGGTTCAGCGTT 162
                                                          M  N  T  P  A  T  L  P  L  G  G
 163  CGGCTGCTCTCCACGGCAATCCGCTGCCCCGGGTGGGCACCCCGAAGCATGAACACGCCTGCAACTCTGCCCCTGGGGGGC 243
       E  D  T  T  W  T  P  G  I  N  A  S  W  A  P  D  E  E  E  D  A  V  R  S  D  G  T
 244  GAGGACACCACCTGGACCCCTGGGATCAACGCCAGCTGGGCTCCGGATGAGGAGGAGGATGCAGTGCGGTCCGACGGCACA 324
       G  T  A  G  M  V  T  I  Q  C  I  Y  A  L  V  C  L  V  G  L  V  G  N  A  L  V  I
 325  GGGACAGCGGGCATGGTAACTATCCAGTGCATCTATGCGCTCGTGTGTCTGGTGGGCCTGGTAGGAAACGCCCTGGTCATA 405
       F  V  I  L  R  Y  A  K  M  K  T  A  T  N  I  Y  L  L  N  L  A  V  A  D  E  L  F
 406  TTCGTGATCCTACGCTATGCCAAAATGAAGACAGCCACCAACATCTACCTGCTCAACCTGGCCGTCGCTGATGAGCTCTTC 486
       M  L  S  V  P  F  V  A  S  A  A  A  L  R  H  W  P  F  G  A  V  L  C  R  A  V  L
 487  ATGCTCAGTGTGCCATTTGTGGCCTCGGCGGCTGCCCTGCGCCACTGGCCGTTCGGGGCGGTGCTGTGCCGCGCAGTGCTT 567
       S  V  D  G  L  N  M  F  T  S  V  F  C  L  T  V  L  S  V  D  R  Y  V  A  V  V  H
 568  AGTGTGGACGGCCTTAACATGTTCACGAGTGTCTTCTGCCTCACAGTGCTCAGCGTGGATCGCTATGTGGCTGTAGTGCAC 648
       P  L  R  A  A  T  Y  R  R  P  S  V  A  K  L  I  N  L  G  V  W  L  A  S  L  L  V
 649  CCTCTGCGAGCTGCCACCTACCGGCGGCCCAGCGTGGCCAAGTTAATCAACCTGGGAGTGTGGCTAGCATCCTTGCTGGTC 729
       T  L  P  I  A  V  F  A  D  T  R  P  A  R  G  G  E  A  V  A  C  N  L  H  W  P  H
 730  ACCCTGCCCATCGCAGTCTTCGCTGACACTAGGCCAGCTCGTGGGGGTGAGGCAGTAGCTTGCAACCTGCACTGGCCTCAC 810
       P  A  W  S  A  V  F  V  I  Y  T  F  L  L  G  F  L  L  P  V  L  A  I  G  L  C  Y
 811  CCGGCCTGGTCTGCAGTCTTTGTGATCTATACTTTTTTGCTGGGCTTCCTACTCCCGGTTCTGGCTATCGGATTATGTTAC 891
       L  L  I  V  G  K  M  R  A  V  A  L  R  A  G  W  Q  Q  R  R  R  S  E  K  K  I  T
 892  CTGCTTATCGTGGGCAAGATGCGTGCTGTGGCCCTGCGGGCTGGCTGGCAACAACGGAGACGCTCAGAGAAGAAGATCACT 972
       R  L  V  L  M  V  V  T  V  F  V  L  C  W  M  P  F  Y  V  V  Q  L  L  N  L  F  V
 973  AGGCTCGTGCTAATGGTGGTGACTGTCTTTGTGCTATGCTGGATGCCATTCTATGTAGTGCAGCTTCTGAATCTGTTTGTC 1053
       T  S  L  D  A  T  V  N  H  V  S  L  I  L  S  Y  A  N  S  C  A  N  P  I  L  Y  G
1054  ACCAGCCTCGATGCCACTGTCAACCATGTGTCCCTCATCCTCAGCTATGCCAACAGCTGTGCCAACCCGATTCTCTATGGT 1134
       F  L  S  D  N  F  R  R  S  F  Q  R  V  L  C  L  R  C  C  L  L  E  T  T  G  G  A
1135  TTCCTCTCAGACAACTTCCGACGCTCTTTCCAGCGGGTTCTGTGCCTGCGCTGCTGTCTCCTGGAAACAACTGGAGGTGCT 1215
       E  E  E  P  L  D  Y  Y  A  T  A  L  K  S  R  G  G  P  G  C  I  C  P  P  L  P  C
1216  GAGGAAGAGCCCCTGGACTACTATGCTACTGCTCTCAAAAGCAGAGGTGGCCCAGGATGCATATGCCCTCCATTGCCCTGC 1296
       Q  Q  E  P  M  Q  A  E  P  A  C  K  R  V  P  F  T  K  T  T  T  F  *
1297  CAGCAGGAGCCCATGCAAGCAGAACCTGCCTGCAAGCGAGTCCCTTTCACCAAGACCACTACTTTTCTGAAAACCATTTCAC 1377
1378  CCTCCCTCAGCCCACCTGCAAGCAGGTCTGCACCACACTCTCAAGCCAGCAACTTCAAGAAAACTCCTGTTGTCACTAAGC 1458
1459  CAGGCCCTTTCAGCAGCCTGTGTTCTGTCCCTAGGAGCCTCAGGACTCCTGCTAGCCCCTGCCTCTCCTAGGACTGACTGG 1539
1540  CTCCAAGGACAACTCCGTGGG  1560
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FIG. 1. Restriction map (*A*) and nucleotide and deduced amino acid sequence (*B*) of the SSTR-4 rat genomic clone. Stippled bar in *A* represents coding region. Single-letter amino acid code is used.

The solution of dissolved cells was transferred to a polypropylene tube, each well was washed with an additional 0.25 ml of 0.1 M NaOH, and the combined solutions were assayed for radioactivity. Competition binding data were analyzed with the LIGAND program (28).

RNA Extraction. Total RNA was isolated from dissected tissue blocks by using developed methods (29). Briefly, tissue was homogenized with a Polytron tissue homogenizer in a buffer containing 4 M guanidine isothiocyanate, 50 mM Tris (pH 7.5), 10 mM EDTA (Fisher Scientific), 1% *N*-lauroylsarcosine (Sigma), and 1% 2-mercaptoethanol (Boehringer Mannheim). The samples were then extracted twice with phenol/chloroform, ethanol-precipitated, subjected to DNase digestion, reextracted with phenol/chloroform, then chloroform-extracted, and ethanol-precipitated. RNA concentrations were estimated based on absorbance at 260 nm.

Poly(A)$^+$ RNA was isolated from hippocampus total RNA using the Micro-Fast Track mRNA isolation kit (Invitrogen).

Preparation of Probes. For nuclease-protection analysis, a ^{32}P-labeled cRNA probe was transcribed in the presence of T3 polymerase from a 343-bp *Sac* I–*Pst* I fragment digested from pSSTR4/BS subcloned into pBluescript II KS(+) after linearization with *Sac* I. The resulting 414-base transcript contained 71 bases of vector sequence and 343 bases of rat (r) SSTR-4 sequence containing the region from the second to the fifth transmembrane domain (see Fig. 1*A*).

A ^{32}P-labeled antisense RNA for RNA analysis was transcribed in the presence of T7 polymerase after linearization of pSSTR4/BS with *Kpn* I.

Nuclease-Protection Assay. Two or five micrograms of total RNA from each tissue was coprecipitated with 50,000 cpm of ^{32}P-labeled rSSTR-4 RNA probe prepared as described above. Samples were resuspended in hybridization buffer containing 40 mM 1,4-piperazine diethanesulfonic acid (pH 6.8)/72% (vol/vol) formamide/1 mM EDTA/0.4 M NaCl, boiled for 3 min, and hybridized at 45°C for 14–16 hr. After hybridization, samples were subjected to combined RNase A and T_1 digestion of nonhybridized probe at 30°C for 1 hr. Stable hybrids were ethanol-precipitated, denatured, and separated on 8% polyacrylamide/8 M urea gels. The dried gel was apposed to Kodak X-Omat x-ray film (Eastman Kodak) to generate an autoradiograph (29).

RNA Hybridization. Poly(A)$^+$ RNA was separated on a denaturing 2.2 M formaldehyde/1% (wt/vol) agarose gel and transferred onto GeneScreenPlus (New England Nuclear) membrane. The membrane was then dried and baked *in vacuo* at 80°C for 2 hr. Blots were hybridized with 2×10^6 cpm of ^{32}P-labeled rSSTR-4 per ml in 50% (vol/vol) formamide/5× SSPE/5× Denhardt's solution/1% SDS/denatured salmon sperm DNA at 100 μg/ml at 60°C overnight. The final washing of blots was in 0.1× SSPE/0.1% SDS at 65°C for 20

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rSSTR4    .......... ..MNTPATLP LGGEDTTWTP GINASWAPDE EEDAVRSDGT   38
mSSTR1    MFPNGTASSP SSSPSPSPGS CGEGACSRGP GSGAADGMEE PGRNASQNGT   50
mSSTR2    .......... ....MEMSSE QLNGSQVWVS SPFDLNGSLG PSNGSNQTEP   36
rSSTR2    .......... ....MELTSE QFNGSQVWIP SPFDLNGSLG PSNGSNQTEP   36

                                  TM1
rSSTR4    ...GTAGMVT IQCIYALVCL VGLVGNALVI FVILRYAKMK TATNIYLLNL   85
mSSTR1    LSEGQGSAIL ISFIYSVVCL VGLCGNSMVI YVILRYAKMK TATNIYILNL  100
mSSTR2    YYDMTSNAV. LTFIYFVVCV VGLCGNTLVI YVILRYAKMK TITNIYILNL   85
rSSTR2    YYDMTSNAV. LTFIYFVVCV VGLCGNTLVI YVILRYAKMK TITNIYILNL   85
                     **  **  *** **  **  ********* * **** ***
              TM2                                        TM3
rSSTR4    AVADELFMLS VPFVASAAAL RHWPFGAVLC RAVLSVDGLN MFTSVFCLTV  135
mSSTR1    AIADELLMLS VPFLVTSTLL RHWPFGALLC RLVLSVDAVN MFTSIYCLTV  150
mSSTR2    AIADELFMLG LPFLAMQVAL VHWPFGKAIC RVVMTVDGIN QFTSIFCLTV  135
rSSTR2    AIADELFMLG LPFLAMQVAL VHWPFGKAIC RVVMTVDGIN QFTSIFCLTV  135
          * **** **   **      *  *****   * * *  **  *  ***  ****
                                                TM4
rSSTR4    LSVDRYVAVV HPLRAATYRR PSVAKLINLG VWLASLLVTL PIAVFADTRP  185
mSSTR1    LSVDRYVAVV HPIKAARYRR PTVAKVVNLG VWVLSLLVIL PIVVFSRT.A  199
mSSTR2    MSIDRYLAVV HPIKSAKWRR PRTAKMINVA VWCVSLLVIL PIMIYAGLRS  185
rSSTR2    MSIDRYLAVV HPIKSAKWRR PRTAKMINVA VWGVSLLVIL PIMIYAGLRS  185
           * *** *** **   *  ** *  **  *   **  **** * **
                                                      TM5
rSSTR4    ARGGEAVACN LHWPHPA..W SAVFVIYTFL LGFLLPVLAI GLCYLLIVGK  233
mSSTR1    ANSDGTVACN MLMPEPAQRW LVGFVLYTFL MGFLLPVGAI CLCYVLIIAK  249
mSSTR2    NQWGRS.SCT INWPGESGAW YTGFIIYAFI LGFLVPLTII CLCYLFIIIK  234
rSSTR2    NQWGRS.SCT INWPGESGAW YTGFIIYAFI LGFLVPLTII CLCYLFIIIK  234
                *     *     *    *  * *   *** *   *  ***  *  *
                                                TM6
rSSTR4    MRAVALRAGW QQRRRSEKKI TRLVLMVVTV FVLCWMPFYV VQL....LNL  279
mSSTR1    MRMVALKAGW QQRKRSERKI TLMVMMVVMV FVICWMPFYV VQL....VNV  295
mSSTR2    VKSSGIRVGS SKRKKSEKKV TRMVSIVVAV FIFCWLPFYI FNVSSVSVAI  284
rSSTR2    VKSSGIRVGS SKRKKSEKKV TRMVSIVVAV FIFCWLPFYI FNVSSVSVAI  284
                *    *  ** *  *  *  ** * *  ** ***
                                TM7
rSSTR4    FVTSLDATVN HVSLILSYAN SCANPILYGF LSDNFRRSFQ RVLCLRCCLL  329
mSSTR1    FAEQDDATVS QLSVILGYAN SCANPILYGF LSDNFKRSFQ RILCL..SWM  343
mSSTR2    SPTPALKGMF DFVVILTYAN SCANPILYAF LSDNFKKSFQ NVLCL....V  330
rSSTR2    SPTPALKGMF DFVVILTYAN SCANPILYAF LSDNFKKSFQ NVLCL....V  330
                     ** *** ******** * *****  ***   ***

rSSTR4    ETTGGAEEEP LDYYATALKS RGGPGCICPP LPCQQEPMQA EPACKRVPFT  379
mSSTR1    D...NAAEEP VDYYATALKS RA.....YSV EDFQPENLES GGVFRNGTCA  385
mSSTR2    KVSGTEDGER SD.......S KQDKSRLNET TETQRTLLNG DLQTSI ...  369
rSSTR2    KVSGAEDGER SD.......S KQDKSRLNET TETQRTLLNG DLQTSI ...  369
                *   *       *                *

rSSTR4    .KTTTF  384
mSSTR1    SRISTL  391
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FIG. 2. Comparison of the deduced amino acid sequence of the rat SSTR-4 with those of the mouse SSTR-1 and the mouse and rat SSTR-2. Sequences were aligned by using the PILEUP function of Genetics Computer Group software (25). Identical amino acids are denoted by asterisks. Putative membrane-spanning domains are indicated by TM1–TM7 and solid bars.

min. Blots were exposed at −80°C to x-ray film with an intensifying screen.

RESULTS AND DISCUSSION

PCR and molecular cloning techniques in conjunction with expression analysis were used to determine the primary structure of the brain-specific somatostatin receptor rSSTR-4. Two sets of sequence-specific oligonucleotide primers directed toward mouse SSTR-1 and SSTR-2 were synthesized and used to amplify sheared rat genomic DNA. Each primer pair amplified a 700-bp fragment of DNA that was subcloned into pBluescript II KS(+) and sequenced. The rat DNAs generated by PCR displayed 100% identity to the mouse clones (18). The rat SSTR-1 and SSTR-2 DNA fragments were then used as probes to screen 7.5×10^5 recombinants of a rat genomic library. Under low-stringency hybridization conditions, 20 positive clones were identified, of which 10 remained hybridized under high stringency (subsequently identified as rat SSTR-1; unpublished work). One of the 10 low-stringency clones, the 15-kb clone rSSTR-4, that displayed sequences homologous to both probes was isolated. A 4.5-kb *Kpn* I–*Eco*RI fragment was sequenced and shown to contain a 1152-bp open reading frame uninterrupted by introns. Fig. 1 shows the restriction map and the nucleotide and deduced amino acid sequence of the genomic clone. The open reading frame encodes a protein of 384 amino acids with a predicted size of 42,087 Da.

As shown in Fig. 2, rSSTR-4 shows a great deal of sequence homology to SSTR-1 and SSTR-2. The amino acid sequence of rSSTR-4 is 60% and 48% identical to that of the mouse SSTR-1 and mouse and rat SSTR-2, respectively (SSTR-1 and SSTR-2 share 46% identity). As expected, rSSTR-4 shares the structural characteristics of receptors of the G protein-receptor superfamily, including a hydrophobic seven-transmembrane-segment structure separated by stretches of hydrophilic residues (30). In addition, the predicted protein sequence has two consensus sites for N-linked glycosylation in the NH_2-terminal region and has serine and threonine residues in the third cytoplasmic loop and COOH-terminal region that could serve as substrates for protein kinases (30). Comparison of the amino acid sequences of the three somatostatin receptors reveals that regions sharing most homology are the membrane-spanning domains. However, sequence divergences among the three receptors may be implicated in the diverse biological effects of somatostatin mediated through a variety of cellular effector systems.

To examine the ligand-binding characteristics of rSSTR-4, a construct containing the complete open reading frame was inserted into the mammalian expression vector pCDNA1 and was used in transient transfection assays using COS-1DM cells. Forty-eight hours after transfection, competition experiments with somatostatin analogs using ^{125}I-labeled SS-14 as radioligand were done. Fig. 3 shows that ^{125}I-labeled SS-14 binding to rSSTR-4-transfected COS-1DM cells was inhibited by SS-14, SS-28, and [D-Trp^8]SS-14 over a concentration range of 10^{-10}–10^{-7} M; IC_{50} values were 4.5, 7.5, and 10.0 nM, respectively. In contrast, the synthetic somatostatin analogs SMS 201-995, IM 4-28, and MK-678 failed to displace ^{125}I-labeled SS-14 binding to transfected cells (Fig. 3). Reubi (14) and Tran *et al.* (15) were among the first to suggest the existence of multiple brain somatostatin receptor subtypes. They observed biphasic displacement by SMS 201-995 of specific ^{125}I-labeled SS-14 binding to receptor preparations from brain but not from pituitary or pancreas. Subsequently, IM 4-28, another cyclic octapeptide SS-14 analog, could not compete for cerebral cortex ^{125}I-labeled SS-14 binding; however, the peptide could bind with high affinity to pituitary homogenates (16). Further evidence for the existence of brain-specific somatostatin receptors was found with the

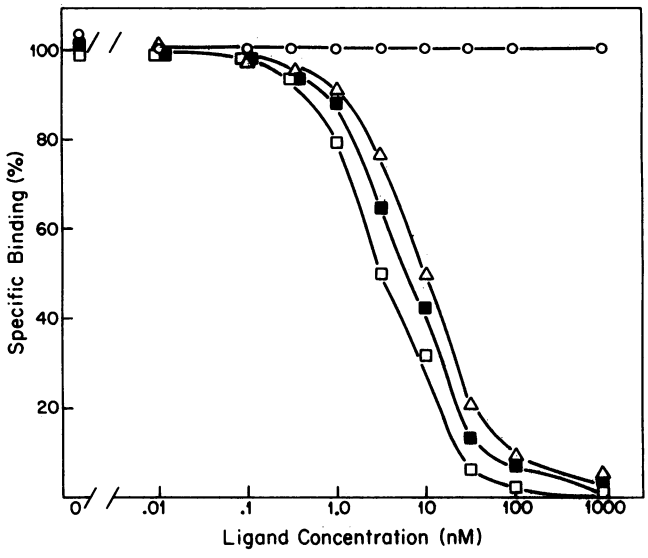

FIG. 3. Pharmacological specificity of ^{125}I-labeled SS-14 binding to COS-1DM cells expressing rSSTR-4. Whole cells transfected with the receptor cDNA were incubated with ^{125}I-labeled SS-14 and the indicated concentrations of unlabeled SS-14 (□), SS-28 (■), [D-Trp^8]SS-14 (△), or SMS 201-995, IM 4-28, or MK-678 (○). Data are expressed as the percentage of radioligand bound in the absence of inhibitor and are mean values of three independent determinations. In all experiments, nonspecific binding represented <15% of the total ^{125}I-labeled SS-14 bound.

cyclic hexapeptide analog MK-678. Competition by MK-678 for ^{125}I-labeled SS-14 binding sites in cortex was incomplete, relative to displacement of the radioligand from hypophysial sites (17). Based on the displacement data obtained in the present study, rSSTR-4 shows binding characteristics—i.e., insensitivity to several conformationally restricted somatostatin analogs—that conform to the brain-specific somatostatin receptor.

Tissue expression of rSSTR-4 mRNA was examined by nuclease-protection assay and RNA blot analysis. For nuclease-protection assay, a 414-base cRNA probe corresponding to 343 bases of rSSTR-4 and 71 bases of vector sequence was used to hybridize with RNA isolated from various tissues (Fig. 4*A*). In marked contrast to RNA blotting and solution hybridization/nuclease-protection studies that demonstrate SSTR-1 and SSTR-2 expression in human and rat central and peripheral tissues (ref. 18 and unpublished work), only RNA prepared from certain brain regions and not from other tissues contained a protected 343-base mRNA (indicating sequence identical to rSSTR-4). The relative abundance of the 343-base-protected fragment in brain correlates well with the density of somatostatin receptor-binding sites reported—i.e., greatest in cortex and hippocampus, moderate in hypothalamus and olfactory bulb, and none detectable in cerebellum (5). RNA blot analysis with polyadenylylated RNA isolated from hippocampus revealed mRNA-hybridizing-band species of 6000 and 2400 nucleotides (Fig. 4*B*). Although the nature of the two RNA species was not analyzed, multiple transcripts could either arise from use of distinct promoters or polyadenylylation sites or could represent transcripts from homologous atypical somatostatin receptor genes.

In summary, the present study describes structural and functional properties of rSSTR-4, a somatostatin receptor that has the pharmacologic properties of a brain-specific somatostatin receptor. Within the central nervous system, somatostatin exerts diverse physiological actions that play a significant role in extrapyramidal motor system and cognitive function (31, 32). In addition, somatostatin exerts profound effects on neural firing and regulates ionic conductances (33,

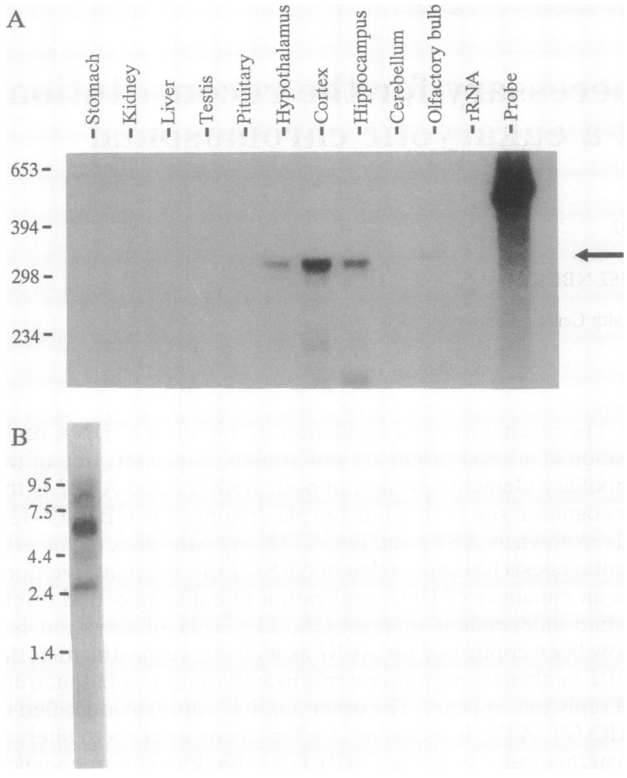

FIG. 4. Tissue distribution of the mRNA encoding rat SSTR-4. (*A*) For nuclease-protection analysis, 2 μg of total RNA from the hippocampus or 5 μg of total RNA from the tissues indicated was hybridized with the coding-region probe. After nuclease digestion, the protected species were analyzed by PAGE and autoradiographed. Arrow indicates the 343-base-protected fragment. The minor bands seen migrating faster than the major protected fragment probably result from hybridization to partially degraded RNA and overdigestion of protected mRNA because the appearance of these bands varied from preparation to preparation. The lane marked rRNA represents a negative control, showing the absence of hybridization of rSSTR-4 complementary RNA probe with *Escherichia coli*-derived ribosomal RNA. The lane designated Probe indicates the mobility of rSSTR-4 complementary RNA probe undigested by RNase. Size markers are indicated by the number of bases, as designated by ^{35}S-3′ end-labeled DNA (M_r marker VI, Boehringer Mannheim). We note that no signal was obtained in negative tissues with as much as 20 μg of total RNA. (*B*) For the RNA blot, 10 μg of poly(A)$^+$ RNA isolated from hippocampus was denatured, electrophoresed on 1% agarose gel, transferred to nylon, and hybridized with a ^{32}P-labeled rSSTR-4 probe. Standards for the RNA blot were a 0.24- to 9.5-kb RNA ladder (GIBCO/BRL).

34). Moreover, brain somatostatinergic systems have been shown to be disturbed and implicated in the motor and cognitive abnormalities associated with Huntington chorea and Alzheimer disease (35, 36). The cloning of rSSTR-4, a brain-specific somatostatin receptor, and the recent cloning of two additional somatostatin receptors also present in the brain provide molecular probes that should lead to identification of the cellular effector systems coupled to these receptors, as well as identification of specific subtypes responsible for mediating the diverse biological effects of somatostatin.

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