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Biochemistry

Expression cloning of a rat brain somatostatin receptor cDNA

[somatostatin 14/somatostatin 28/SMS-(201–995)/G-protein-coupled receptor]

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ABSTRACT **We have used an expression-cloning strategy to isolate a cDNA encoding a somatostatin (somatotropin release-inhibiting factor, SRIF) receptor from rat cortex and hippocampus. A positive clone was identified by autoradiography after binding of radiolabeled SRIF to COS-1 cells previously transfected with pools of cDNA clones. The deduced amino acid sequence of the receptor displays sequence and structural homology to the family of G-protein-coupled receptors. The affinity of various SRIF analogs to the expressed receptor resembles their effects on growth hormone release from pituitary cells. In addition, the distribution of the mRNA in various tissues corresponds to that described for native SRIF receptors. Therefore, we conclude that we have isolated a rat brain SRIF receptor cDNA.**

Somatostatin (somatotropin release-inhibiting factor, SRIF), a neuropeptide initially isolated from the hypothalamus, was shown to act on many different cell types by inhibiting the secretion of hormones, including growth hormone (GH), insulin, glucagon, gastrin, and secretin (1–3). In addition, SRIF was shown to act as a neurotransmitter (4–6). Therefore, SRIF has widespread functions as a modulator of neuronal activity as well as endocrine and exocrine secretion. These regulatory effects are mediated by specific membrane receptors on the SRIF target tissues (7). High-affinity, saturable binding sites have been demonstrated in cortex, hippocampus, substantia nigra, pituitary gland, pancreas, adrenal cortex, and several tumor types—for instance, endocrine, breast, lung, or brain tumors (8–11). Radiolabeled SRIF analogs have been used extensively to characterize the binding properties of SRIF receptors on the various target tissues. Previously, it has been shown that SRIF receptors are coupled to GTP-binding proteins (G proteins) (12–15). Pretreatment of SRIF receptor-positive cells with pertussis toxin abolished the ability of SRIF receptors to interact with cellular effector systems, indicating the involvement of a pertussis toxin-sensitive G protein (16, 17). The results of previous studies have suggested the existence of at least two SRIF receptor types (18–20). They exert similar affinities for the native peptides somatostatin 14 (SRIF-14) and somatostatin 28 (SRIF-28) but different affinities for synthetic SRIF analogs.

In the present study we describe the isolation and primary structure of a SRIF receptor cDNA, the pharmacology of the expressed receptor, and the distribution of the mRNA.* The approach chosen for cloning of the receptor cDNA included the construction of a cDNA expression library with RNA isolated from the developing rat cortex and hippocampus, transfection of pools of clones into COS-1 cells, and identification of cells expressing SRIF receptors by autoradiography after binding of iodinated ligands (21, 22).

MATERIALS AND METHODS

cDNA Cloning. RNA was purified from cortex and hippocampus dissected from brains of 6-day-old rats by the guanidinium thiocyanate/acid phenol method (23). Poly(A)$^+$ RNA was enriched by two subsequent passages over an oligo(dT)-cellulose column as described (24). Five micrograms of poly(A)$^+$ RNA was used in the synthesis of oligo(dT)-primed double-stranded cDNA using the Amersham cDNA synthesis system. After addition of phosphorylated *Dra* III adapters (pGTC GAC CAC CTC and pGTG GTC GAC) the cDNAs were size-selected by gel filtration [Sepharose CL-2B (Pharmacia), 39 × 1.5 cm column; running buffer = 1 M KCl/35 mM Tris·HCl, pH 8.3/30 mM KH_2PO_4/1 mM EDTA; flow rate, 8 ml/hr; fraction volume, 2 ml]. Fractions were concentrated and desalted using Centricon-100 ultrafiltration devices (Amicon), previously preabsorbed with yeast tRNA. The first fraction, eluting with the void volume as determined with dextran blue, was subsequently ligated into *Dra* III-digested pXMD1. We constructed this vector from pXMT3 (25) to reduce vector recirculation. Two oligonucleotides (pAAT TCA CGA GGT GAT TCG CGA ATC ACC TCG TGC TCG AGA and pAAT TTC TCG AGC ACG AGG TGA TTC GCG AAT CAC CTC GTG) were inserted into the *Eco*RI restriction site of pXMT3. This results in the integration of two asymmetric *Dra* III cleavage sites separated by a short spacer and the restoration of one of the *Eco*RI restriction sites. For the cloning, the spacer segment was removed after *Dra* III cleavage.

Electroporation of the ligation products into *Escherichia coli* strain MC1061 (26) yielded 8.5 × 10^6 independent clones with an average insert size of 2.5 kilobases (kb) and >90% inserts. Amplification of the cDNA library was performed on agar plates to compensate for different growth rates.

Isolation of a cDNA Clone Encoding a SRIF-14 Receptor. Plasmid DNA from pools of 800–1000 independent clones was introduced into COS-1 (27) cells by the DEAE-dextran procedure (28). COS-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. The cells were seeded 1 day before transfection at a density of about 7000 cells per cm^2. On the next day the cells were washed twice with DMEM and incubated with 0.4 mg of DEAE-dextran per ml, 1.25 μg of plasmid DNA per ml, and 10% NuSerum in DMEM. After 4 hr at 37°C, the cells were washed once with DMEM, incubated for 2 min in 10% (vol/vol) dimethyl sulfoxide in calcium/magnesium-free phosphate-buffered saline, again washed with DMEM, and incubated for 2–3 days in normal cell culture medium containing 15 μg of gentamycin per ml. Positive pools were identified by incubating the cells with 60 pM of ^{125}I-labeled SRIF-14 (^{125}I-SRIF-14; Amersham) and 60 pM of ^{125}I-labeled [Tyr^3]SMS-(201–995) {^{125}I-[Tyr^3]SMS-(201–995)} (20, 29) [labeled by the chloramine-T iodination

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Abbreviations: SRIF, somatotropin release-inhibiting factor, somatostatin; GH, growth hormone.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M93273).

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procedure (30) to a specific activity of about 2000 Ci/mmol; 1 Ci = 37 GBq] in 50 mM Hepes-KOH, pH 7.4/1 mM $MnCl_2$/0.5 μg of bacitracin per ml/100 kallikrein inhibitor units of aprotinin per ml/0.1% bovine serum albumin (31, 32) for 1 hr at room temperature. Specific binding in this buffer was higher than in buffers containing NaCl or other salts at physiological concentrations (data not shown). To reduce background staining, the radiolabeled ligands were centrifuged for 1–2 hr at 4°C at 60,000 rpm in a SW 60 Ti rotor prior to the binding assay. After two washes with the same buffer lacking the radioligands, the cells were dried and the rims of the tissue culture plates were removed. For the autoradiography, Kodak X-Omat AR films were exposed at −80°C for 1–3 days.

Due to stability problems of the recombinant plasmids in *E. coli* strain MC 1061, we subsequently transferred DNA from positive plasmid pools into *E. coli* strain HB101. A single colony was identified by SIB selection as described (33).

Other Recombinant DNA Techniques. All DNA manipulations were performed using standard techniques (24). The sequence was analyzed with the DNASIS and PROSIS computer programs (Pharmacia LKB). All plasmid DNAs were purified by ion-exchange chromatography on Quiagen columns (Diagen, Düsseldorf, F.R.G.). For the blotting, RNAs were separated on a 1% agarose gel containing 2.2 M formaldehyde (24) and transferred to a Hybond N (Amersham) membrane using a pressure blotter (Stratagene). After UV-crosslinking of the RNA to the membrane with a Stratalinker (Stratagene), the RNA was hybridized to a single-stranded DNA probe generated by an asymmetric PCR with an oligonucleotide primer located between putative transmembrane regions IV and V (34). Hybridization was performed at 55°C in 50% formamide/50 mM sodium phosphate, pH 6.5/0.1% SDS/5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 2.5× Denhardt's solution/1 mM EDTA/200 μg each of herring sperm DNA and yeast RNA per ml. The membrane was washed twice at room temperature in 2× SSC/0.1% SDS and twice at 65°C in 0.1× SSC/0.1% SDS.

Pharmacology. For the binding experiments, the isolated cDNA clone was introduced into COS-1 cells by electroporation (26, 35). Briefly, the cells were removed from the plates by trypsin treatment, collected by centrifugation, washed once with calcium/magnesium-free PBS, and finally resuspended at a concentration of 1×10^7 cells per ml. Aliquots (500 μl) of the cell suspension were incubated for 5 min at room temperature with 10 μg of plasmid DNA in a 0.4-cm cuvette (Bio-Rad). A pulse was applied at 1.2 kV, 25 μF (Gene Pulser, Bio-Rad) and, after 10 min on ice, the cells were diluted with DMEM supplemented with 10% fetal calf serum and 15 μg of gentamycin per ml and plated. Two days later the cells were scraped off the plates and membranes were prepared as follows: the transfected COS-1 cells were homogenized in cold 10 mM Hepes (pH 7.6) and centrifuged at 20,000 × *g* for 20 min, the pellet was washed twice with 20 ml of the same buffer, and the protein concentration was determined. COS-1 cell membranes were stored at −70°C. A time course of specific ^{125}I-[Tyr^3]SMS-(201–995) binding to COS-1 cell membranes showed that the amount of specific binding increased with time, reaching a plateau after 60 min, which was stable up to 120 min (data not shown). For radioligand binding experiments, membranes corresponding to 10 μg of protein per tube were incubated for 60 min at room temperature with the indicated concentration of ^{125}I-[Tyr^3]SMS-(201–995), a well-characterized specific ligand for SRIF receptors (20, 29), or ^{125}I-SRIF-14 in the presence of 5 mM $MgCl_2$, 20 μg of bacitracin per ml, 0.5% bovine serum albumin, and 10 mM Hepes (pH 7.5). Incubation was stopped by rapid filtration through Whatman GF/C filters. Filters were washed two times with 5 ml of cold 10 mM Hepes (pH 7.5). Specific radioligand binding was defined as total binding minus binding in the presence of 0.1 μM SMS-(201–995) or SRIF-14 (nonspecific binding), which was <20% of the total binding.

Competition experiments were performed by incubating the membranes with increasing concentrations of unlabeled SRIF analogs. Binding curves were calculated from three experiments (triplicate determinations) using the computer-fitting program of De Lean (36).

RESULTS

Identification of a Rat Brain SRIF Receptor cDNA. RNA isolated from cortex and hippocampus of 6-day-old rats was chosen since these brain areas contain a fairly high density of somatostatin receptors. In addition, the receptor number is strongly increasing in these brain areas at this developmental stage (37), implicating an intensive receptor synthesis that may be reflected by a high mRNA concentration.

From 30 pools of recombinants tested, two triggered positive signals in our screening assay. One of them was subsequently split into eight subpools consisting of 200 clones each, of which three were positive. From one of these pools 960 colonies were grown individually in microtiter dishes. All 96 colonies from a given plate were combined for testing. The DNAs isolated from three plates triggered SRIF binding in transfected COS-1 cells. The colonies from one of those plates were subdivided further and found to contain three identical positive clones with inserts of 2.1 kb.

Fig. 1 *A–D* demonstrates the increasing signals that were obtained at the various steps of the purification procedure. The binding of ^{125}I-SRIF-14 and ^{125}I-[Tyr^3]SMS-(201–995) is specific since the addition of 1 μM (each) unlabeled SRIF-14 and SMS-(201–995) prevents binding of the labeled ligands (Fig. 1 *E* and *F*).

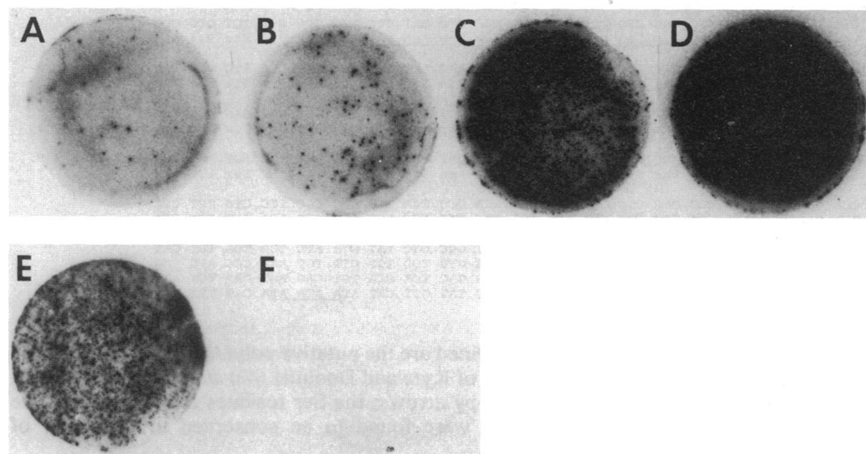

FIG. 1. Autoradiographs obtained during the screening procedure. (*A–D*) Pools of 1000 (*A*), 200 (*B*), 100 (*C*), and 10 (*D*) recombinants were tested for ^{125}I-SRIF-14 and ^{125}I-[Tyr^3]SMS-(201–995) binding activity after transfection into COS-1 cells by the DEAE-dextran procedure. Exposure was for 3 days at −80°C. (*E* and *F*) Binding with the purified receptor clone transfected into COS-1 cells by electroporation. (*E*) Total binding. (*F*) Binding in the presence of 1 μM (each) SRIF-14 and SMS-(201–995). The difference in the number of transfected cells (*D* and *E*) in the upper and lower panels is due to the lower transfection efficiency of electroporation compared to the DEAE-dextran procedure (unpublished results).

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DNA and Deduced Amino Acid Sequence. The 2116-base-pair (bp) cDNA contained an open reading frame of 1107 bp encoding a protein of 369 amino acids. The initiation codon and the surrounding nucleotides closely resembled the optimal consensus sequence (except for a C → A transition in position −4) (38). The poly(A) tail is preceded by an unusual putative poly(A) signal (AAUAAU) (39).

The deduced amino acid sequence predicts a protein of 41.2 kDa; however, from crosslinking experiments it has been estimated that somatostatin receptors isolated from brain have molecular masses of 60–74 kDa (40–42). The difference is probably due to N-linked glycosylation (40). The putative extracellular N-terminal region contains four consensus sequences used for the glycosylation of Asn residues (Fig. 2) (44).

Analysis of the hydropathic character of the encoded protein displays the presence of seven hydrophobic, putatively membrane-spanning regions (indicated in Fig. 2) (44). Thus, the SRIF receptor is a member of the receptor family with seven membrane-spanning regions, which is also suggested by its coupling to a G protein (12–14). A number of amino acid residues that are conserved in this family of receptors are also found in this sequence (Fig. 2). An example is the tripeptide sequence Asp-139 Arg-140 Tyr-141, which is considered important in coupling to G proteins (45). Three Cys residues are found, two of which, Cys-115 and Cys-193, may form a S–S bridge between extracellular loops 1 and 2 (46). The third, Cys-328, may, in analogy to the human β_2-adrenergic receptor, attach the C-terminal region to the membrane via a palmitoyl anchor (47). A number of consensus sites for serine phosphorylation by several protein kinases (casein kinase II, cGMP-dependent protein kinase, multifunctional calmodulin-dependent protein kinase II) (48) have been identified and are depicted in Fig. 2; however, their functional relevance remains to be elucidated.

Tissue Distribution. Fig. 3 depicts an RNA blot on which RNAs were hybridized with a single-stranded DNA probe. Two intensively labeled bands corresponding to RNAs of 2.3 and 2.6 kb were visible. These RNAs are present in cortex, hippocampus, the exocrine pancreatic cell line AR4-2J (50), pituitary, and a pancreas tumor (51) but not in kidney, a tissue devoid of SRIF receptors (unpublished data). The highest density of SRIF receptor mRNA was found in AR4-2J cells. The lanes containing cortex or hippocampus poly(A)$^+$ RNAs displayed additional weak bands of 3–3.5 kb and ≈6 kb.

Saturation Experiments. The concentration dependence of ^{125}I-[Tyr^3]SMS-(201–995) binding is shown in Fig. 4. The radioligand exhibited high-affinity binding to membranes of transfected COS-1 cells. Membranes from COS-1 cells transfected with plasmid only or with plasmids containing unrelated inserts displayed no binding to either ligand (data not shown). The binding data were best fitted by a one-site model indicating the presence of a single class of high-affinity binding sites with an affinity constant (K_d) of 51 ± 2 pM and the maximal number of binding sites (B_{max}) of 157 ± 5 fmol/mg of protein ($n = 2$). Obviously, this B_{max} refers to the whole cell population, whereas only about 15% of the cells are transfected by electroporation (unpublished data). Scatchard transformations of the saturation curves (Fig. 4 *Inset*) were linear, again demonstrating the presence of a single class of high-affinity binding sites for ^{125}I-[Tyr^3]SMS-(201–995) in the transfected cells. Similar experiments using ^{125}I-SRIF-14 also resulted in high-affinity binding (K_d = 104 pM).

Competition Experiments. To further characterize the structural requirements of somatostatin binding to the cloned receptor expressed in COS-1 cells, we performed competition experiments with various SRIF analogs using ^{125}I-[Tyr^3]SMS-(201–995) and ^{125}I-SRIF-14 as radioligands. The compounds tested inhibited specific radioligand binding in a monophasic manner. The rank order of potency was consis-

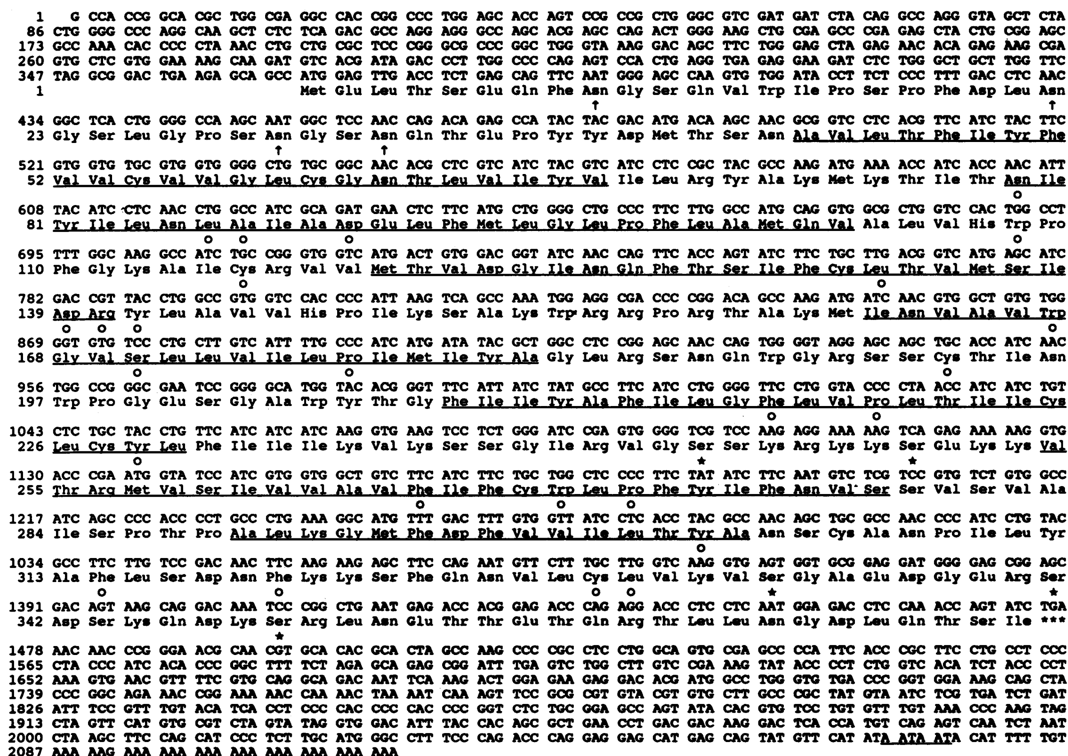

FIG. 2. Nucleotide and deduced amino acid sequence of the cloned SRIF receptor. Underlined are the putative poly(A) signal in nucleotide positions 2071–2076 and the putative membrane-spanning regions defined using the algorithm of Kyte and Doolittle (43) at a window setting of 20 amino acids. The Asn residues possibly subjected to N-linked glycosylation are marked by arrows; the Ser residues that are part of the recognition sequence for different protein kinases are indicated by stars. Residues that were found to be conserved in a number of G-protein-coupled receptors are emphasized by open circles.

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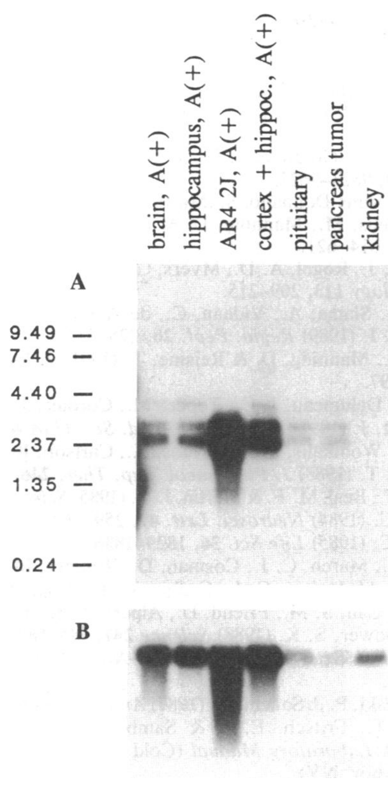

FIG. 3. (*A*) Tissue distribution of the mRNA encoding the cloned SRIF receptor. Twenty micrograms of total RNA or, where indicated, 5 μg of poly(A) RNA [A(+)] was analyzed (see text). The brain and hippocampus RNAs were from 14-day-old rats, the cortex plus hippocampus RNAs were from 6-day-old rats, and the pituitary and kidney RNAs were from adult rats. Positions of size standards are indicated in kb. Exposure time was 2 days at −80°C. (*B*) After removing the SRIF receptor probe, the same membrane was hybridized with a human β-actin probe (49); exposure was for 2 hr at room temperature.

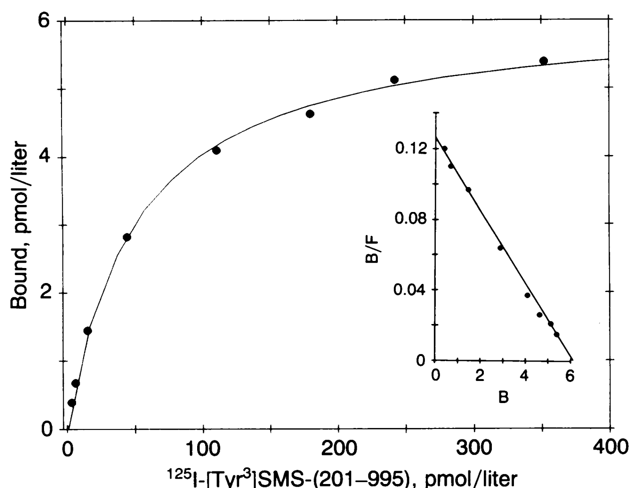

FIG. 4. Concentration dependence of ^{125}I-[Tyr^3]SMS-(201–995) binding to membranes prepared from COS-1 cells transfected with the SRIF receptor cDNA. Membranes were incubated with the indicated concentrations of radioligand in the absence and, to determine unspecific binding, presence of 0.1 μM SMS-(201–995). Data are mean values of three determinations and are expressed as pmol of specific binding per liter. (*Inset*) Binding data plotted by the method of Scatchard. The maximal binding capacity (B_{max}) was calculated from the intercept on the abscissa (B_{max} = 155 fmol/mg of protein) and the affinity from the slope of the line (K_d = 52 pM). B/F, bound/free.

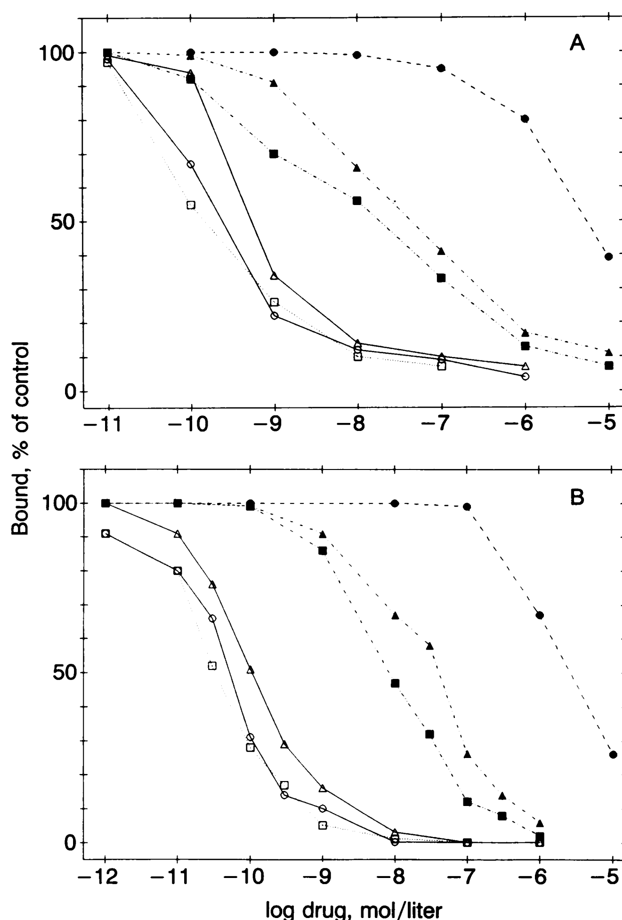

FIG. 5. (*A*) Concentration dependence for the inhibition of ^{125}I-SRIF-14 binding by SRIF analogs. Membranes prepared from COS-1 cells transfected with the receptor cDNA were incubated with ^{125}I-SRIF-14 and the indicated concentrations of SRIF-28 (□), SRIF-14 (○), SMS-(201–995) (△), [Lys-ol^8]SMS-(203–304) (▲), [Orn^5]SMS-(204–354) (■), and [citrulline^5]SMS-(217–717) (●). Results are expressed as percentage of the maximal specific binding observed in the absence of competitor. In all experiments the nonspecific binding was <20% of the total binding. Data are mean values of three determinations. (*B*) A similar experiment as in *A* using ^{125}I-[Tyr^3]SMS-(201–995) as the labeled ligand.

tent with their ability to inhibit GH release from pituitary cells (29). The analogs [Lys-ol^8]SMS-(203–304) and [Orn^5]SMS-(204–354), which are weakly active on inhibition of GH release, also exhibited weak affinities to the SRIF receptors on the membranes of transfected COS-1 cells. [Citrulline^5]-SMS-(217–717), which is nearly inactive in the GH release assay and has only weak affinity for cortex membrane receptors, did not show any activity in our binding experiments up to a concentration of 0.1 μM. In contrast, SRIF-28, SRIF-14, and SMS-(201–995) very effectively inhibited radioligand binding with IC_{50} values in the subnanomolar range. Representative competition curves obtained with the various SRIF analogs and radioligands ^{125}I-[Tyr^3]SMS-(201–995) and ^{125}I-SRIF-14 are shown in Fig. 5 *A* and *B*. An excellent correlation was found between the ability of these peptides to inhibit GH release from pituitary cells and to displace the ^{125}I-SRIF-14 binding from membranes of transfected COS-1 cells (r = 0.98, P < 0.01).

It is interesting to note that SRIF-28 always displayed the highest affinity of the SRIF receptors expressed in COS-1 cells. The inhibitory concentration for half-maximal inhibition (IC_{50}) was calculated to be $1–2 \times 10^{-10}$ mol/liter using SRIF-28 as competitor, which is in good agreement with the previously reported affinity of SRIF-28 to its receptor (30).

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DISCUSSION

With an expression-cloning strategy we have isolated a cDNA clone encoding a SRIF receptor from a cDNA library constructed with mRNA isolated from the developing rat cortex and hippocampus. The deduced amino acid sequence contains seven putative membrane-spanning regions and several amino acids that are conserved in other members of the same gene family. The distribution of the receptor mRNA was analyzed by RNA blotting. Two strongly hybridizing RNAs were detected in brain and pituitary tissues as well as in a pancreatic tumor and a pancreas-derived cell line. This distribution resembles that described for somatostatin receptors (1–3). We have not yet analyzed the nature of the two RNAs. It is possible that they represent two independent albeit strongly cross-hybridizing mRNAs. Two arguments render this unlikely. (*i*) Both RNAs are equally distributed in all tissues analyzed, which would not be expected for two independent mRNAs. (*ii*) Hybridization of a similar blot with a RNA probe derived from the 3′ noncoding region of the cloned cDNA also labeled both bands (data not shown). Therefore, it seems more likely that the two bands are produced by the alternative use of poly(A) sites. With the RNAs isolated from cortex or hippocampus, additional weak bands (at 3–3.5 kb and ≈6 kb) are visible on the blots, which may, in fact, represent homologous mRNAs encoding other SRIF receptor types. These bands were not visible after hybridization with the RNA probe, which may, however, be attributed to a relatively high background created by this probe.

The binding of SRIF and its analogs was examined using membranes derived from transfected COS-1 cells transiently expressing SRIF receptors. When membranes from these cells were incubated with either ^{125}I-SRIF-14 or ^{125}I-[Tyr^{3}]SMS-(201–995), specific high-affinity binding could be detected. The receptors are comparable in their pharmacological specificity to those already characterized in pituitary cells. We demonstrated for several analogs an excellent correlation between the ability to displace radioligand binding from receptors expressed in COS-1 cells and their inhibitory activity on GH release from pituitary cells (29). Specific binding of either ^{125}I-SRIF-14 or ^{125}I-[Tyr^{3}]SMS-(201–995) could be displaced by SRIF-14 as well as SRIF-28 and SMS-(201–995), indicating that there is no selectivity of the cloned receptor for either ligand.

Based on these results we conclude that we have indeed cloned the cDNA that encodes the most commonly studied SRIF receptor type. The receptor is a member of the family of G-protein-coupled receptors with seven transmembrane regions. The pharmacological profile of this receptor when expressed in the heterologous COS-1 cell system is indistinguishable from native SRIF receptors, and the tissue distribution closely resembles that of the described SRIF binding sites.

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