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Molecular cloning and pharmacological characterization of the rat sigma$_1$ receptor

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Abstract

In an effort to further understand the pharmacology of sigma receptors, we have cloned the rat homolog of the sigma$_1$ receptor. We isolated a cDNA clone (rs2–2) from rat brain tissue using reverse transcriptase–polymerase chain reaction (RT–PCR) and 5′ and 3′ rapid amplification of cDNA ends (RACE) that encoded a full-length sequence of 223 amino acids. The predicted protein sequence of the clone has high homology with that of the murine (93.3%), guinea pig (93.7%), and human (96%) sigma$_1$ receptors. Northern analysis showed a major mRNA band of approximately 1.8 kb. RT–PCR revealed the presence of the mRNA in all the tissues tested, with high levels in the brain, spinal cord, liver, thymus, adrenal glands, and kidneys. When expressed in Chinese hamster ovary (CHO) cells, the level of sigma$_1$ binding increased markedly, and the binding profile was consistent with sigma$_1$ sites. However, measurable levels of sigma$_1$ binding present in the cell lines before transfection made the interpretation of these results difficult. To ensure that the binding reflected the transfected protein, we tagged the receptors with a hemagglutinin (HA) epitope at the amino terminus and examined binding in immunoprecipitated receptors. Western analysis using an antisera against the HA epitope revealed a molecular weight of ~28 kDa, close to the predicted value. The receptor binding profile of the immunopurified receptor was consistent with that seen with traditional sigma$_1$ binding sites. Thus, rs2–2.HA encodes a high-affinity [^{3}H](+)-pentazocine binding site with characteristics of a rat sigma$_1$ receptor.

Keywords: Sigma$_1$ receptor; Rat; Cloning; Anti-opioid; Pentazocine

1. Introduction

Sigma receptors were originally proposed based upon the actions of the benzomorphan opiate (±)-SKF-10047 [1]. However, the pharmacology of (±)-SKF-10047 is complex and involves more than one receptor class. The sites defined as sigma today are not opioid and represent a unique type of receptor. Two subtypes of sigma receptors have been proposed based upon binding profiles, with the sigma$_1$ receptor displaying high affinity for (+)-pentazocine and haloperidol [2]. Sigma receptors have been observed across many species and are present at high levels in the CNS, the immune system, and the liver [3–7]. Within the CNS, the distribution of sigma receptors is heterogeneous [6]. Pharmacologically, sigma$_1$ receptors comprise a potent anti-opioid analgesic system [8–14]. Administered with opioid analgesics, however, sigma$_1$ receptor agonists, such as (+)-pentazocine, have markedly diminished analgesic potency. These systems also appear to be tonically active, since sigma$_1$ antagonists enhance opioid analgesia. Although sigma$_1$ receptors modulate analgesia mediated through all the opioid receptors, kappa analgesics are most affected by tonic sigma$_1$ activity. Furthermore, the level of this activity varies among strains of mice.

The sigma$_1$ receptor was first cloned from guinea pig liver [15]. Based on the homology with the guinea pig sigma$_1$ cDNA, human [16] and mouse [17] sigma$_1$ receptors have also been cloned. The structure of the sigma$_1$ receptor shows no homology with opioid receptors or other known mammalian proteins. Its predicted amino acid sequence suggests two transmembrane domains, demonstrating that it does not correspond to a traditional G-protein-coupled receptor. Understanding the molecular actions of this receptor

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Abbreviations: DTG, *N*,*N*′-di(*o*-tolyl-guanidine); HA, hemagglutinin; RT–PCR, reverse transcriptase–polymerase chain reaction; RACE, rapid amplification of cDNA ends; and CHO, Chinese hamster ovary.

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is a major goal toward establishing its function(s). However, differences at the level of the cDNA encoding the receptor among species can complicate molecular studies on the receptor. The rat has been used extensively to evaluate the neuropharmacology of many varieties of drugs. To explore the sigma$_1$ receptor function at a molecular level in rats, we have now cloned the rat homolog of the sigma$_1$ receptor.

2. Materials and methods

[^{3}H](+)-Pentazocine was purchased from the New England Nuclear Corp. (+)-Pentazocine and (−)-pentazocine were gifts from the Research Technology Branch of NIDA. DTG and CHAPS were purchased from Research Biochemicals International. Anti-HA monoclonal antibody was purchased from BabCO. Fetal bovine serum was purchased from BioWhittaker. Antibiotics and other media were purchased from GIBCO. All other chemicals were purchased from the Sigma Chemical Co.

Glass fiber filters (No. 32) for receptor binding were purchased from Schleicher & Schuell. Formula 963 liquid scintillant was purchased from the New England Nuclear Corp.

2.1. Cloning the full-length rat sigma$_1$ receptor using 5′ and 3′ RACE

A partial sequence of a rat sigma$_1$ receptor was obtained through RT–PCR, using primers based upon the cloned mouse homolog (M39SE: CGG TGG GCA TGG ATC ACC CTG and M229AN: CCC AGA GCC CTG AAT GCC AGC), and was sequenced. Three internal antisense primers were designed based upon this partial sequence. The 5′ and 3′ RACE kits were purchased from Boehringer Mannheim. The first strand cDNA was synthesized from rat brain and liver total RNA (ULTRASPEC^{TM} RNA isolation system, Biotecx) using the specific sigma$_1$ internal primer SP1 and AMV (avian myeloblastosis virus) reverse transcriptase. The reactions were incubated for 1 hr at 55° and for 10 min at 65°. The resulting cDNAs were purified further and tailed by terminal transferase (dA-tailed cDNA). PCR fragments were generated by amplifying these dA-tailed cDNAs using an oligo dT-anchor primer and the internal gene specific primer SP2 and cloned into TOPO TA vector using TOPO TA cloning kits (Invitrogen). The cloned cDNAs were sequenced in our laboratory and by Cornell University Sequencing Core Facility. The full-length rat sigma$_1$ receptor was isolated using RT–PCR and a set of primers designed from the RACE product. To avoid the artifact of PCR products, we did three replicates for each RT reaction and PCR reaction. All of the nine PCR products were cloned and sequenced. Only one mutation was seen in a single clone. One of the clones, rs2–2 (GenBank Accession Number AF067769), was sequenced in both directions, using appropriate oligonucleotide primers.

2.2. Expression of the rat sigma$_1$ receptor

CHO cells (obtained from the ATTC) were maintained in tissue culture flasks in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals). Cells were grown in a 6% CO_2 and 94% air humidified atmosphere at 37°. Plates of cells were used when 75–95% confluent. Cells were lifted for subculturing after a 5-min incubation at 37° in 5 mL of PBS containing trypsin.

The parent plasmids were restriction digested with *Kpn*I and *Eco*RI and subcloned into the *Kpn*I-*Eco*RI site of a cytomegalovirus promoter-based expression vector, pcDNA3 (Invitrogen), and transformed into bacteria. The epitope-tagged constructs were generated by PCR. PCR fragments containing an HA tag sequence at the N-terminus of the full-length rs2–2 were first amplified using the sense primer containing the HA sequence and 5′ end sequence of rs2–2 and the antisense primers designed from the untranslated region at the 3′ end. The PCR fragments were subcloned into the above expression vector, and the resulting plasmids, rs2–2/pcDNA3 and rs2–2.HA/pcDNA3, were transfected into CHO cells using LipofectAMINE PLUS^{TM} (GIBCO).

Cells were harvested 48 hr after the transfection, washed twice with cold PBS, and lifted in 15 mL PBS. After pelleting, the cells were resuspended in 20 vol. of Tris buffer [50 mM Tris (pH 7.7) at 25°, 100 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and homogenized. The homogenates were incubated at 25° for 15 min and centrifuged at high speed (49,000 *g*) for 30 min. The pellets were resuspended in 0.32 M sucrose and either used immediately or frozen down at –70° until used. The prepared membranes could be kept frozen for several weeks without significant loss of sigma$_1$ binding activity. The protein concentrations were determined by the method of Lowry *et al.* [18].

For immunoprecipitation, membranes were solubilized with CHAPS (3 mM) in potassium phosphate buffer (10 mM; pH 7.4) containing EDTA (1 mM) for 2 hr. The mixture was centrifuged at 13,000 *g* for 40 min, and the supernatants were incubated with an anti-HA monoclonal antibody (1:50 dilution) for 2 hr. The mixture was further incubated with protein G-Sepharose for 2 hr, and the pellets were separated by centrifugation at 500 *g*. The pellets were washed twice with solubilization buffer, twice with the buffer without detergent, and then resuspended with potassium phosphate buffer (10 mM, pH 7.4) for use in binding assays. For western blots, the pellets were directly resuspended with SDS gel sample buffer [62.5 mM Tris–HCl (pH 6.8), 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol] and run on polyacrylamide gel electrophoresis. All of the procedures were performed at 0–4°.

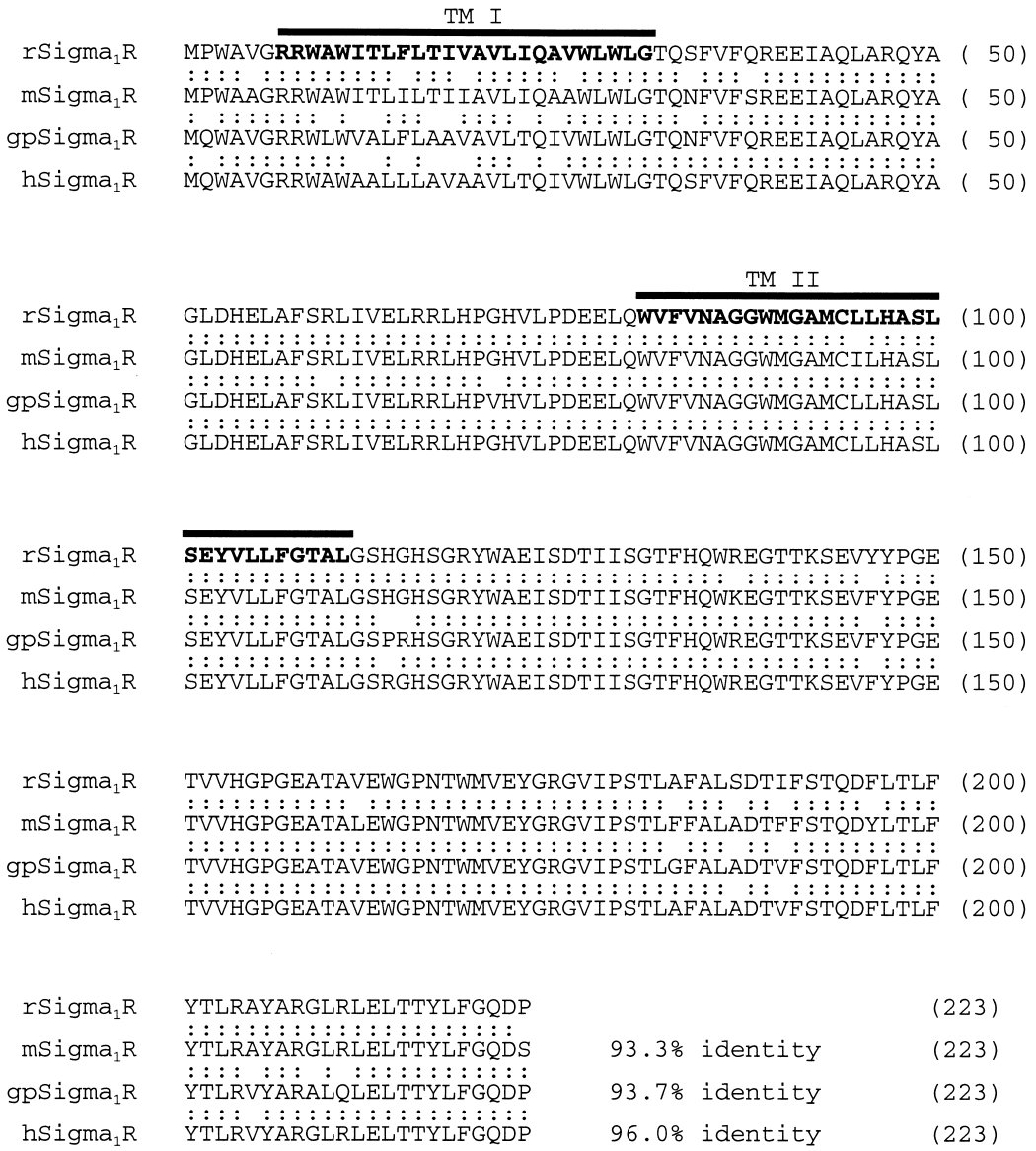

Fig. 1. Predicted amino acid sequence of the rat sigma$_1$ receptor. The predicted amino acid sequence of the rat sigma$_1$ receptor (rS1R) is presented and aligned with murine (mS1R), guinea pig (gpS1R), and human (hS1R) sigma$_1$ receptors. Note: the letters set in bold type and the lines over the sequences correspond to the predicted transmembrane domains. The rat sigma$_1$ receptor cDNA sequence has been submitted to GenBank (Accession No. AF067769).

2.3. [^{3}H](+)-pentazocine binding assays

All binding assays were performed at 37° for 3 hr with 1-mL aliquots of membranes (0.1 mg/mL) suspended in potassium phosphate buffer (10 mM, pH 7.2; 1 mL) with [^{3}H](+)-pentazocine (2 nM, except for saturation studies that used concentrations ranging from 1 to 15 nM). After the incubation, each sample was diluted with 3 mL of ice-cold Tris buffer (5 mM, pH 7.7, at 25°) and filtered over glass-fiber filters (Schleicher & Schuell). Filters were washed twice with 6 mL of buffer and counted. After filtering, the filters were counted in 5 mL of scintillation fluor (5 mL) after sitting for at least 4 hr. All experiments were performed in triplicate and replicated at least three times. Specific binding was determined by subtracting the nonspecific binding, defined as the binding remaining in the presence of haloperidol (1 μM) from total binding. K_D and K_i values were calculated by nonlinear regression analysis (Prism, GraphPad Software).

Binding to immunoprecipitated receptors was performed on the beads at 37° for 3 hr. The bound and free ligands were separated by centrifugation at 500 *g* for 5 min at room temperature. The pellets were washed once with binding buffer and counted in scintillation fluor.

2.4. Data analysis

Non-linear regression analysis of binding was performed using the program Prism (GraphPAD Software). K_i values were calculated as: $K_i = (IC_{50})/(1 + S)$, where S = (con-

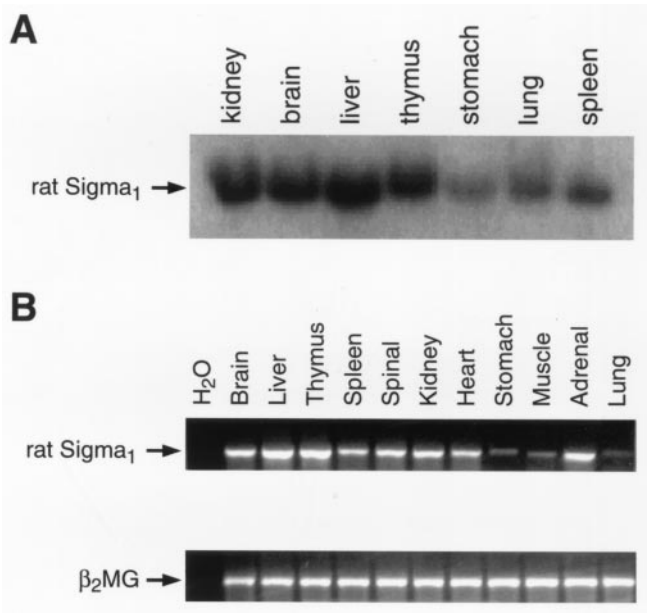

Fig. 2. RT–PCR and northern analysis. (A) Northern blotting. Total RNA isolated from rat tissues (kidney, brain, liver, thymus, stomach, lung, and spleen) was analyzed by using a ^{32}P-labeled rs2–2 fragment as previously described [17]. Results are a representative experiment that has been replicated twice. (B) RT–PCR. Total RNA was isolated from rat tissues. Sigma receptor cDNAs were synthesized using RT–PCR reactions as described in "Materials and methods." Lane 1, H_2O; 2, brain; 3, liver; 4, thymus; 5, spleen; 6, spinal cord; 7, kidney; 8, heart; 9, stomach; 10, muscle; 11, adrenal gland; and 12, lung. Internal sample loading controls were shown with β_2 microglobulin in the lower panel. Results are a representative experiment that has been replicated twice.

centration of radioligand)/(K_D of radioligand) [19,20]. Results are presented as means ± SEM of triplicate experiments.

3. Results

3.1. Cloning of the rat sigma$_1$ receptor

The clone isolated using RT–PCR and 5′ and 3′ RACE, rs2–2, had a predicted protein sequence of 223 amino acids and was highly homologous to that of the mouse (93.3% identity) [17], guinea pig (93.7% identity) [15], and human (96.0% identity) [16] sigma$_1$ receptors (Fig. 1). The predicted amino acid sequence using Kyte–Doolittle analysis suggested two potential transmembrane domains, similar to the other clones and consistent with a membrane protein.

Table 1
Saturation studies of [^{3}H](+)-pentazocine in rs2–2 transfected and non-transfected CHO cells

Cells	K_D (nM)	B_{max} (pmol/mg protein)
CHO	2.1 ± 0.2	2.32 ± 0.14
Vector/CHO	2.0 ± 0.1	2.25 ± 0.09
rs2–2/CHO	2.3 ± 0.1	21.8 ± 0.58
rs2–2.HA/CHO	2.2 ± 0.1	20.3 ± 0.29

Binding was measured with [^{3}H](+)-pentazocine (0.5 to 15 nM) on the membranes prepared from the indicated cells as described in "Materials and methods", except for the rs2–2.HA/CHO samples in which binding was performed after solubilization and immunoprecipitation. K_D values were determined from saturation studies. Results are means ± SEM of three independent determinations.

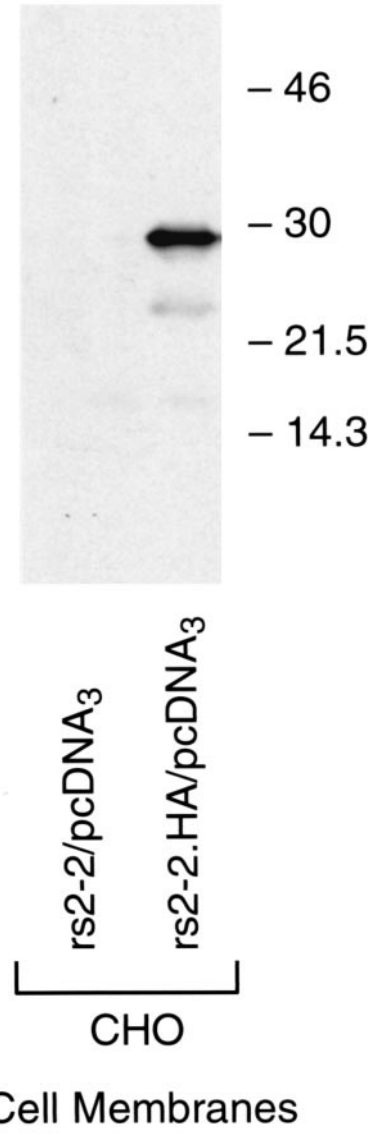

Fig. 3. Western analysis. Membranes from cells transiently transfected with rs2–2.HA were subjected to SDS–PAGE, were transferred to nitrocellulose membranes, and then were analyzed by a western blot, using an antibody against HA as previously described [17]. The western blot yielded a band with a size of approximately 28 kDa.

The rat sigma$_1$ receptor is widely distributed throughout the body. Northern analysis with a full-length cDNA probe against total RNA (Fig. 2A) and RT–PCR performed using the total RNA isolated from various tissues showed the sigma$_1$ mRNA in all tissues examined, particularly the brain, liver, thymus, spinal cord, kidneys, and adrenal glands (Fig. 2B). However, the levels did differ. The inten-

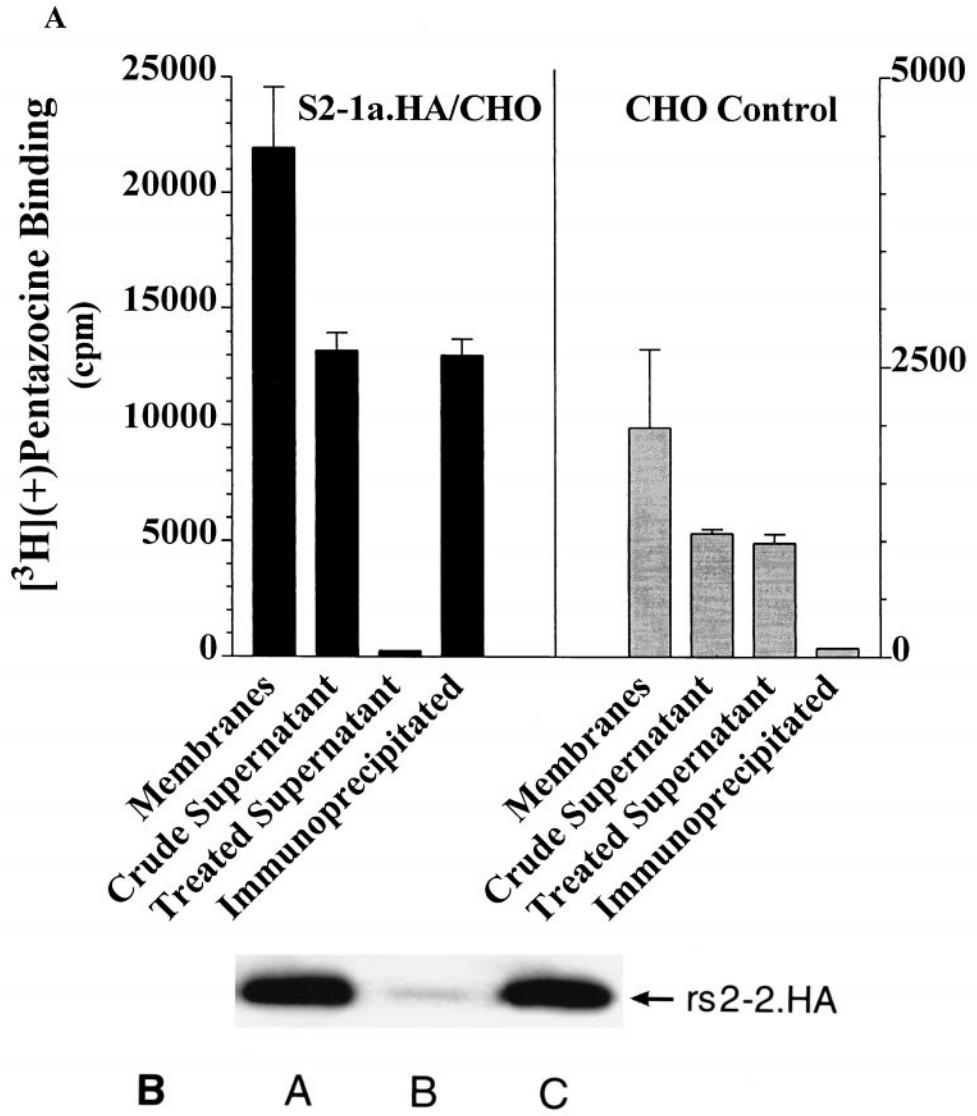

Fig. 4. Receptor binding and western blotting of the transfected and immunoprecipited sigma receptors. (A) Specific [^{3}H](+)-pentazocine binding was performed with different membrane fractions of rs2–2.HA transfected CHO cells (left panel) or nontransfected CHO control cells (right panel) as described in "Materials and methods." Binding was first assessed in the membrane fractions. The membranes were then solubilized with CHAPS, as described in "Materials and methods," and the crude supernatant was examined for binding. The transfected receptors were then immunoprecipitated, and binding was assessed in the pellet and the treated supernatant. Note the differences in the scales on the y-axis for the transfected and nontransfected cell lines. (B) Western blotting of rs2–2.HA transfected CHO cells was performed as described in "Materials and methods." Lane A, crude solubilized cell membrane before immunoprecipitation; Lane B, supernatants after immunoprecipitation; and Lane C, immunoprecipitated pellets.

sity of the bands on the northern blots for stomach, lung, and spleen were clearly lower than the other tissues, and PCR results also showed lower levels in stomach, muscle, and lung.

3.2. Characterization of the cloned rat sigma$_1$ receptor

(+)-Pentazocine is a selective sigma$_1$ ligand, whereas DTG binds to sigma$_2$ sites [21]. Although haloperidol binds to both sigma subtypes, it has higher affinity for the sigma$_1$ receptor [2,5,6,22,23]. First, we transiently expressed the rat sigma$_1$ receptor clone (rs2–2) in CHO cell lines and examined [^{3}H](+)-pentazocine binding (Table 1). The K_D value in the transfected cells was 2.3 nM with a B_{max} approximately 10-fold greater than in control cells. Transfection of the cells with the vector alone gave binding values identical to those in nontransfected cells. Thus, the up-regulation of binding in the cell line expressing rs2–2 was not simply due to transfection.

However, the presence of natively expressed sigma$_1$ receptors in the control CHO cell lines still complicated the interpretation of these results. To ensure that we were measuring binding to the protein encoded by rs2–2, we next transfected CHO cells with an HA-tagged rs2–2 clone. The addition of the HA-epitope allowed us to immunoprecipitate

Table 2
Competition of [^{3}H](+)-pentazocine binding in rs2–2 transfected CHO cells

Competitors	K_i (nM)		
	Vector	rs2–2.HA	Immunoprecipitated rs2–2.HA
(+)-Pentazocine	1.9 ± 0.6	2.5 ± 0.6	2.2 ± 0.2
(−)-Pentazocine	115 ± 14	18 ± 2.3	29 ± 2.0
Haloperidol	5.5 ± 0.6	2.5 ± 0.7	1.6 ± 0.1
DTG	29 ± 7.2	104 ± 23	103 ± 7.0

Competition studies against [^{3}H](+)-pentazocine binding were performed with at least four concentrations of ligand in membranes prepared from CHO cells transiently transfected with the vector alone or rs2–2.HA, as described in "Materials and methods." Competition studies also were performed by using immunoprecipitated receptors from rs2–2.HA/CHO cells. After transient transfection of CHO cells with rs2–2.HA, the membranes were solubilized and the receptors were immunoprecipitated with an HA antibody, as described in "Materials and methods." Results are the means ± SEM of three independent determinations.

the transfected receptor and separate it from the endogenous binding sites, which did not have the HA tag. In this way, we could be certain that we were examining binding to the protein encoded by the rat sigma$_1$ clone, rs2–2.

Western blotting of solubilized membranes from CHO cells expressing rs2–2.HA with an antibody against the HA epitope revealed a protein of about 28 kDa, close to the predicted molecular weight (Fig. 3). We then assessed the effectiveness of immunoprecipitation by examining on western blots the solubilized cell membranes before immunoprecipitation and both the supernatant and the pellet after immunoprecipitation. Immunoprecipitation isolated almost all of the HA-tagged receptor (Fig. 4). We then assessed binding in control CHO cells and rs2–2.HA transfected CHO cells. Binding in the rs2–2.HA transfected cells was almost 10-fold greater than in the control CHO cells in the solubilized membrane fraction. As expected, immunoprecipitation brought down virtually all of the [^{3}H](+)-pentazocine binding. Very little binding remained in the supernatant after immunoprecipitation. Conversely, the binding in the nontransfected CHO cells remained in the supernatant after immunoprecipitation, with almost no binding present in the immnoprecipitated fraction.

Competition studies confirmed the high affinity of both (+)-pentazocine and haloperidol for the cloned receptor, whereas DTG and (−)-pentazocine were less potent (Table 2). Thus, the cloned receptor has characteristics consistent with a sigma$_1$ receptor.

4. Discussion

Sigma$_1$ receptors are part of a potent anti-opioid system. Sigma$_1$ agonists greatly reduce opioid analgesia, while antagonists potentiate opioid actions by eliminating the tonic sigma receptor activity. Yet our understanding of sigma receptors remains limited. Rats are well suited to explore the pharmacological role of sigma$_1$ receptors. To examine the role of sigma$_1$ receptors at the molecular level, we have isolated and cloned the rat homologue of the sigma$_1$ receptor. Its structure is closely related to the murine [17], guinea pig [15], and human [16] homologues. The clone has been expressed in CHO cell lines, and its pharmacological properties have been examined. The binding profile of the rat clone expressed in CHO cell lines is consistent with the traditional pharmacology of sigma$_1$ receptors and similar to the binding profile we observed in the cloned murine sigma$_1$ receptor. The high affinity of both (+)-pentazocine and haloperidol, coupled with the low affinity of DTG, indicates that the clone encodes a sigma$_1$ receptor.

All of the cell lines we have examined in the past have sigma binding. Although the 10-fold increase in binding following transfection with rs2–2 and rs2–2.HA strongly implied that the clones were encoding the sigma$_1$ receptor, the presence of sigma binding sites in nontransfected cell lines made it difficult to prove that the increased binding was actually due to expression of the rs2–2 clone. By tagging the clone with an HA epitope, we were able to demonstrate conclusively that the clone encoded the binding site.

Although we now have the structure of the sigma$_1$ receptor, we are still uncertain as to its functions at the molecular level. It does not correspond to any traditional class of receptors. However, having the structure of the receptor will enable us to explore the pharmacological relevance and behavioral functions of this receptor, which in turn may eventually lead to the elucidation of its actions at the molecular level.

Acknowledgments

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