Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8072–8077, July 1996
Pharmacology

Purification, molecular cloning, and expression of the mammalian sigma$_1$-binding site

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Communicated by James Black, King's College School of Medicine and Dentistry, London, United Kingdom, April 18, 1996 (received for review March 1, 1996)

ABSTRACT **Sigma-ligands comprise several chemically unrelated drugs such as haloperidol, pentazocine, and ditolylguanidine, which bind to a family of low molecular mass proteins in the endoplasmic reticulum. These so-called sigma-receptors are believed to mediate various pharmacological effects of sigma-ligands by as yet unknown mechanisms. Based on their opposite enantioselectivity for benzomorphans and different molecular masses, two subtypes are differentiated. We purified the sigma$_1$-binding site as a single 30-kDa protein from guinea pig liver employing the benzomorphan (+)[^{3}H]pentazocine and the arylazide (−)[^{3}H]azidopamil as specific probes. The purified (+)[^{3}H]pentazocine-binding protein retained its high affinity for haloperidol, pentazocine, and ditolylguanidine. Partial amino acid sequence obtained after trypsinolysis revealed no homology to known proteins. Radiation inactivation of the pentazocine-labeled sigma$_1$-binding site yielded a molecular mass of 24 ± 2 kDa. The corresponding cDNA was cloned using degenerate oligonucleotides and cDNA library screening. Its open reading frame encoded a 25.3-kDa protein with at least one putative transmembrane segment. The protein expressed in yeast cells transformed with the cDNA showed the pharmacological characteristics of the brain and liver sigma$_1$-binding site. The deduced amino acid sequence was structurally unrelated to known mammalian proteins but it shared homology with fungal proteins involved in sterol synthesis. Northern blots showed high densities of the sigma$_1$-binding site mRNA in sterol-producing tissues. This is also in agreement with the known ability of sigma$_1$-binding sites to interact with steroids, such as progesterone.**

The concept of sigma-receptors was introduced by W. R. Martin and coworkers (1, 2), who differentiated the unique psychomimetic effects of norallylmetazocine (SKF10,047) (sigma-syndrome) from the effects of morphine (μ-syndrome) and ketocyclazocine (K-syndrome) (3). This concept changed gradually, and at present sigma-receptors are defined by their ability to bind several chemically unrelated drugs with high affinity (4, 5). Two different sigma-binding site subtypes (subtypes 1 and 2) have been proposed based on differences in their drug-binding profile (6). Endogenous sigma-ligands are not known, although progesterone has been suggested to be one of them (7–9). Possible sigma-site-mediated drug effects include modulation of glutamate receptor function, neurotransmitter response, neuroprotection, behavior, and cognition (10–22). Most of these studies implied that sigma-binding sites are plasmalemmal elements of the signal transduction cascade. Drugs reported to be selective sigma-ligands were evaluated as antipsychotics (23–25). Due to the lack of structural and functional information, the pharmacological significance of sigma-binding sites remains enigmatic.

The verapamil-like calcium-antagonists azidopamil (a photoligand) and emopamil (an antiischemic drug) are also high-affinity sigma-ligands that were previously employed as specific probes to purify and clone a novel drug-binding membrane protein from liver. This was distinct from the sigma$_1$-binding site, although it showed substantial pharmacological and biochemical similarities with sigma-receptors (26–29). Until now, sigma-ligand studies suffered from the lack of structural information. To clarify its primary structure, we purified the protein carrying the sigma$_1$-binding site and cloned the corresponding cDNA using reverse transcriptase-PCR and degenerate oligonucleotides. Expression in *Saccharomyces cerevisiae* revealed that this cDNA was sufficient to form a high-affinity drug-binding domain with all characteristics of mammalian sigma$_1$-binding sites.

EXPERIMENTAL PROCEDURES

Materials. (+)[^{3}H]Pentazocine (32 Ci/mmol) was obtained from NEN. (−)[^{3}H]Azidopamil (87 Ci/mmol) and the unlabeled phenylalkylamines were kindly provided by Knoll (Ludwigshafen, Germany). Sigma-ligands were a gift of J. Traber (Tropon, Cologne, Germany). The following chemicals were obtained from the indicated sources: opipramol, CIBA–Geigy (Vienna); ceramic hydroxyapatite, Bradford protein reagent, and molecular weight markers, Bio-Rad; Q-, SP-, heparin-, and lysine-Sepharose, Pharmacia; phosphatidylcholine, Avanti Polar Lipids (Alabaster, AL); and all other chemicals, Sigma (Deisenhofen, Germany).

Binding Assays. (+)[^{3}H]Pentazocine binding experiments with membrane-bound and solubilized proteins were carried out by incubating 0.4–2.1 nM (+)[^{3}H]pentazocine with protein in 0.25 or 0.5 ml of 25 mM Tris·HCl (pH 9 at 4°C, giving a final pH of 8.3 at 22°C) for 4–12 h in the absence or presence of 0.3 mg of phosphatidylcholine per ml. Nonspecific binding defined by 1 μM pentazocine was subtracted from total binding to yield specific binding. For inhibition experiments serial drug dilutions were made in dimethyl sulfoxide (DMSO) and added directly to the incubation mixture. The final DMSO concentration was 1% (vol/vol), which did not affect radioligand binding. Separation of bound from free ligand by filtration over GF/C glass fiber filters (Whatman) was carried out as described (26). Binding parameters were calculated by nonlinear curve fitting to a rectangular hyberbola (K_d, B_{max}) or the general dose–response equation (IC_{50}, slope factor) (30). From the IC_{50}-value, the K_i was calculated as described (31). Protein concentrations were determined according to Bradford (32), using BSA as a standard.

Purification. All the purification procedures were carried out at 5°C. Guinea pig liver microsomes were prepared as described (28). After centrifugation for 1 h at 100,000 × g, the

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Abbreviations: $K_xH_yPO_4$, K_2HPO_4/KH_2PO_4; SKF10,047, norallylmetazocine.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z66537).
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microsomal pellet was solubilized in 300 ml 1% (wt/vol) digitonin in buffer A [5% (vol/vol) glycerol/20 mM Tris·HCl, pH 10 (4°C)/0.1 mM phenylmethylsulfonylfluoride] containing 165 mM NaCl at a protein concentration of 5–6 mg/ml. After 1 h incubation at 5°C, this mixture was centrifuged as above. The supernatant was applied (350 ml/h) to a Q-Sepharose column (4.5 × 23.5 cm) equilibrated with 0.1% (wt/vol) digitonin in buffer A. Bound protein was step-eluted with buffer A containing 275 mM or 1 M NaCl, respectively. Activity was recovered only in the 275 mM NaCl step. This fraction was diluted with 3 M K_2HPO_4/KH_2PO_4 ($K_xH_yPO_4$) (pH 7.0) to a final concentration of 0.15 M and applied (300 ml/h) to a hydroxyapatite column (2.5 × 12 cm) equilibrated with 0.1% (wt/vol) digitonin/0.15 M $K_xH_yPO_4$, pH 7.0 in buffer A. Retained binding activity was step-eluted with 140-ml portions of 0.1% (wt/vol) digitonin containing 0.4, 0.6, 0.8, 1.25, and 1.5 M $K_xH_yPO_4$ (pH 8.0). The activity-containing fraction (1.25 M $K_xH_yPO_4$) was dialyzed overnight (molecular weight cutoff of 6000–8000) against 20 mM $K_xH_yPO_4$/4 mM NaCl, pH 6.5. The dialysate was applied (300 ml/h) to a SP-Sepharose column (Pharmacia; 2.5 × 8 cm) equilibrated in buffer B [0.1% (wt/vol) digitonin/20 mM $K_xH_yPO_4$/4 mM NaCl, pH 6.5). Binding activity eluting in the flow-through was collected and applied (55 ml/h) to a lysine-Sepharose column (1.5 × 5 cm). Binding activity was eluted with 60 mM NaCl and passed through (110 ml/h) heparin-Sepharose (1.5 × 7 cm) equilibrated with 60 mM NaCl in buffer B. The activity-containing flow-through was collected and dialyzed overnight (molecular weight cutoff of 6000–8000) against 20 mM $K_xH_yPO_4$, pH 6.5. The dialysate was applied (300 ml/h) to a spermine-agarose column (1.5 × 4 cm) equilibrated in buffer B. The spermine-agarose flow-through was applied (80 ml/h) to thiopropyl-Sepharose (1 × 4 cm) equilibrated with buffer B. Adsorbed binding activity was step-eluted with buffer B containing 3 mM and 15 mM L-cysteine, respectively. The activity-containing fraction was passed through (50 ml/h) Green-Sepharose (Pharmacia; 1 × 1 cm) equilibrated in buffer B. Purified binding activity was eluted with 300 mM NaCl in buffer B.

Photoaffinity Labeling and SDS/PAGE. (−)[^{3}H]Azidopamil was incubated in the dark with the thiopropyl-Sepharose eluate in 0.01% (wt/vol) digitonin/25 mM Tris·HCl, pH 9 (4°C)/0.3 mg of phosphatidylcholine per ml in the absence or presence of drugs for 6 h at 22°C. Samples were then irradiated for 1 min with an ultraviolet lamp (Sylvania–GTE germicide) at 6-cm distance. Photolyzed protein was concentrated and resuspended in SDS-sample buffer (26) containing 10 mM dithiothreitol (reducing conditions) and separated on SDS/polyacrylamide gels as described (26). For fluorography, gels were equilibrated in Amplify (Amersham), dried, and exposed to Kodak X-Omat AR5 films for the indicated times (at −80°C). Ferguson plot analysis of the purified sigma$_1$-binding site was carried out as described (28).

Electroelution, Tryptic Fragmentation, and Amino Acid Sequencing. The thiopropyl-Sepharose eluate was dialyzed (molecular weight cutoff of 12,000–14,000) for 48 h against 0.05% (wt/vol) SDS to remove digitonin. The lyophilized sample was resuspended in SDS/sample buffer containing 10 mM dithiothreitol and separated by preparative SDS/PAGE. After negative staining of the gel with $CuCl_2$ (33), the sigma$_1$-binding polypeptide was cut out from the gel, minced, and electroeluted in a Bio-Rad electroelution apparatus in 0.01% (wt/vol) SDS/25 mM Tris/192 mM glycine for 15 h at 100 V. After electroelution, samples were reduced [1% (vol/vol) 2-mercaptoethanol] in the presence of 4% (wt/vol) SDS, alkylated with iodacetic acid, and dialyzed for 18 h against 6 M urea/10 mM sodium phosphate, pH 7.2, containing 20 g/liter Dowex AG1X2 resin (Bio-Rad). Dialysis was continued for 24 h against 0.05% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/5 mM Tris·HCl, pH 8.5 (4°C). Samples were concentrated ≈20-fold and incubated with 4 μg of *N*-tosyl-l-phenylalanine chloromethylketone-trypsin (final concentration, 80 μg/ml) for 15 h at 37°C. Tryptic fragments were loaded onto a Vydac (Hesperia, CA) C_4 column (2.1 × 150 mm, 35°C) using an Applied Biosystems model 130A separation system. The reversed-phase column was equilibrated with 2% (vol/vol) acetonitrile/5 mM trifluoroacetic acid at a flow rate of 0.1 ml/min. Elution was achieved in the presence of a linear gradient from 2 to 50% (vol/vol) acetonitrile over 70 min at a flow of 0.1 ml/min. The individual peaks were collected directly onto Porton peptide supports (Porton Instruments, Tarzana, CA), which were dried and subjected to automated Edman degradation in a Porton Instruments PI2090E micro-sequencer. Typical repetitive yields during sequencing of the various peptide fragments were 90%. The following unique amino acid sequences were ob-

Table 1. Characterization of (+)[^{3}H]pentazocine binding to microsomal membranes from guinea pig liver and brain and from *S. cerevisiae* cells overexpressing the 6xHIS-tagged sigma$_1$-binding site (6×His-λGP8-ORF)

Compound	Guinea pig liver		Guinea pig brain		Yeast-expressed 6xHIS-λGP8-ORF	
	K_i, nM	Slope factor	K_i, nM	Slope factor	K_i, nM	Slope factor
Haloperidol	0.2 ± 0.1	1.06 ± 0.14	0.2 ± 0.2	0.98 ± 0.12	0.7 ± 0.3	1.23 ± 0.18
(+/−)Pentazocine	1.7 ± 0.1	1.23 ± 0.16	1.5 ± 0.1	1.01 ± 0.01	3 ± 0.4	1.19 ± 0.17
(+)-3-PPP	6 ± 1	0.85 ± 0.06	7 ± 2	0.87 ± 0.03	11 ± 4	1.00 ± 0.12
Ditolylguanidine	14 ± 2	0.89 ± 0.01	19 ± 3	0.88 ± 0.02	43 ± 12	0.95 ± 0.14
Dextrometorphan	30 ± 17	0.82 ± 0.16	44 ± 13	0.96 ± 0.10	59 ± 21	0.89 ± 0.11
(+)-SKF10, 047	41 ± 7	0.84 ± 0.19	41 ± 4	0.92 ± 0.09	44 ± 10	0.88 ± 0.06
(−)-SKF10, 047	1900 ± 330	0.99 ± 0.12	1970 ± 400	1.10 ± 0.07	3070 ± 250	1.08 ± 0.01
Ifenprodil	2 ± 0.5	0.96 ± 0.02	2 ± 0.2	0.97 ± 0.02	5 ± 1	0.90 ± 0.08
Opipramol	0.3 ± 0.1	1.16 ± 0.06	0.2 ± 0	1.03 ± 0.02	0.3 ± 0.2	1.00 ± 0.05
(+)-Emopamil	1.2 ± 0.3	1.15 ± 0.09	1.1 ± 0.3	1.07 ± 0.07	2 ± 1	0.93 ± 0.02
(−)-Emopamil	4 ± 2	0.87 ± 0.13	6 ± 2	0.93 ± 0.11	13 ± 4	0.92 ± 0.05
Azidopamil	42 ± 7	0.95 ± 0.06	58 ± 10	0.92 ± 0.03	160 ± 39	1.12 ± 0.03
Trifluoperazine	15 ± 3	0.97 ± 0.07	21 ± 4	0.92 ± 0.08	46 ± 18	1.08 ± 0.12
Testosterone	1200 ± 50	1.03 ± 0.03	1,400 ± 30	0.96 ± 0.01	2340 ± 40	0.93 ± 0.04
Progesterone	260 ± 10	0.95 ± 0.00	338 ± 8	0.92 ± 0.02	572 ± 2	0.94 ± 0.02
Corticosterone	35,600 ± 300	1.09 ± 0.02	28,000 ± 360	0.90 ± 0.05	49,800 ± 5540	0.90 ± 0.01

Binding assays and data analysis were performed as described. Microsomal protein (1–10 μg/ml) was incubated for 12 h at 22°C with (+)[^{3}H]pentazocine (0.4–0.5 nM) in the absence or presence of seven concentrations of each drug. Nonspecific binding was defined in the presence of 1 μM pentazocine. Data given are means ± SDs (n = 3). The following B_{max} and K_d values were determined by saturation analysis with (+)[^{3}H]pentazocine (n = 3): liver, 11.7 ± 0.7 pmol/mg, 0.8 ± 0.1 nM; brain, 6.0 ± 0.2 pmol/mg, 1.0 ± 0.2 nM; and 6xHIS-λGP8-ORF, 60.7 ± 8.7 pmol/mg, 2.4 ± 0.5 nM. Data shown are means ± SDs (n = 3).

tained: peptide PBP43, ALTLELTTY; peptide PBP45, SEVFYPGETVVHGPGEATAVEWG; peptide PBP47, (N/G)VIP(S/C); and peptide PBP54, YWAEISDTIIS.

Immunological Techniques. A polyclonal antiserum (anti-PBP45) was raised in New Zealand white rabbits against a synthetic peptide corresponding to the amino acid residues of peptide PBP45 with an additional N-terminal lysine residue as described (28). For immunoprecipitation, the (−)[^{3}H]azidopamil-photolabeled microsomal protein was solubilized in 2% (wt/vol) SDS for 30 min. Solubilized protein was diluted 20-fold in RIA buffer [1% (wt/vol) Triton X-100/50 mM NaCl/20 mM Tris·HCl, pH 7.4 (22°C)/0.5 mg of BSA per ml), and insoluble material was removed by centrifugation at 12,500 × *g*. Solubilized microsomes were then added to anti-PBP45 or preimmune immunoglobulins adsorbed to protein A-Sepharose in RIA buffer. After incubation for 12 h at 4°C the protein A-Sepharose was washed five times with 1 ml of RIA buffer, once with 1 ml of 150 mM NaCl/20 mM Tris·HCl, pH 7.4 (22°C) and 30 μl of sample buffer containing 10 mM dithiothreitol were added. Immunostaining of Western blots using affinity-purified anti-PBP45 was performed as described (28).

Radiation Inactivation. Samples were irradiated with 10-MeV electrons from a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD) at a protein concentration of 6–8 mg/ml as described (34). Radiation dose measurements were made with thermoluminescent dosimeters. During irradiation, the samples were maintained at −135°C with a stream of cold nitrogen gas. The irradiated samples were stored at −80°C until assays were performed. Target sizes were calculated by expressing the specific binding activity in irradiated samples (A_D) as a function of binding observed in the nonirradiated controls (A_0) from $\ln(A_D/A_0) = -K \times D$, where D is the dose of radiation [Mrads; (1 rad = 0.01 Gy)]. A direct measure of the target size can be obtained using the following equation: target mass (kDa) = 1792 × −K. Correction of the binding data obtained at a single ligand concentration for the change in K_d was performed as described (35). Glucose-6-phosphatase activity was assayed spectrophotometrically as described (26). The inactivation of glucose-6-phosphatase yielded a molecular mass of 75 ± 17 kDa (n = 9), which was in agreement with previously reported data (75 and 53 kDa respectively; refs. 36–38).

PCR and Molecular Cloning. First strand cDNA was synthesized from guinea pig total RNA with an oligo(dT)primer (first strand cDNA synthesis kit; Pharmacia). The 59-bp PCR product was obtained with two degenerate PCR primers deduced from the peptide PBP45, 5′-GA(A/G)GT(A/T/G/C)TT(T/C)TA(T/C)CC(A/T/G/C)GG-3′ and 5′-AG(A/T/G/C)TG(A/T/G/C)CG(A/T/G/C)CA(A/T/G/C)CG(A/G)AG-3′. Sequencing was performed according to standard procedures. A 20-bp oligonucleotide, 5′-GGGGAGACAGTGGTGCAC-3′, was designed based on the center of the 59-bp PCR product and was used with an oligo(dT)primer (5′-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$-3′) to amplify a 1035-bp cDNA from guinea pig liver. This cDNA was used to screen a λGT10 guinea pig liver cDNA library (29).

Expression in *S. cerevisiae*. Protein expression in *S. cerevisiae* strain JB811 was performed as described (29). A 30-bp oligonucleotide, 5′-AAAAGCTTAAAATGTCCACAATGCTCTTCT-3′, and a 35-bp oligonucleotide, 5′-TTTGCGGCCGCTCAAGGGTCTTGGCCGAAGAGGTA-3′, were designed to amplify the entire open reading frame of clone λGP8 and to introduce restriction enzyme sites for *Hin*dIII and *Not*I, respectively. A 9E10 c-*myc* tag (39) and a 6xHIS tag were introduced at the N terminus using oligonucleotide cassettes containing *Xho*I and *Hin*dIII restriction sites. For spectrophotometric scans of nonsaponifiable lipids (40), cells were collected by centrifugation and resuspended in destilled water. Sterols were extracted from cell samples of ≈10 mg of wet weight.

RESULTS AND DISCUSSION

Characterization of the Sigma$_1$-Binding Site in Guinea Pig Liver Microsomes. To prove that the high-affinity (+)[^{3}H]pentazocine-binding site in guinea pig liver represents the previously described (27) sigma$_1$-binding site, its properties were investigated in detailed equilibrium binding studies. As expected, haloperidol and pentazocine were more potent inhibitors than (+)-propyl-3-(3-hydroxyphenyl)piperidine, ditolylguanidine, and dextromethorphan, and the (+)-enantiomer of the benzomorphan SKF10,047 was of 40-fold higher affinity than the (−)-enantiomer (refs. 41–43; Table 1). Saturation studies with (+)[^{3}H]pentazocine revealed a K_d of 0.8 ± 0.1 nM at pH 8.3. As reported previously (44) for the [^{3}H](+)-propyl-3-(3-hydroxyphenyl)piperidine-binding site in brain membranes, binding was pH-dependent with an optimum in Tris buffer at pH > 9.3. This was due to an increase of the K_d from 0.6 ± 0.1 nM (n = 2) at pH 9.3 to 2.7 ± 0.1 nM (n = 2) at pH 7.3 without changes in B_{max} (12.0 ± 0.1, n = 2 versus 12.0 ± 0.4 pmol per mg of protein, n = 2). Our data demonstrate that

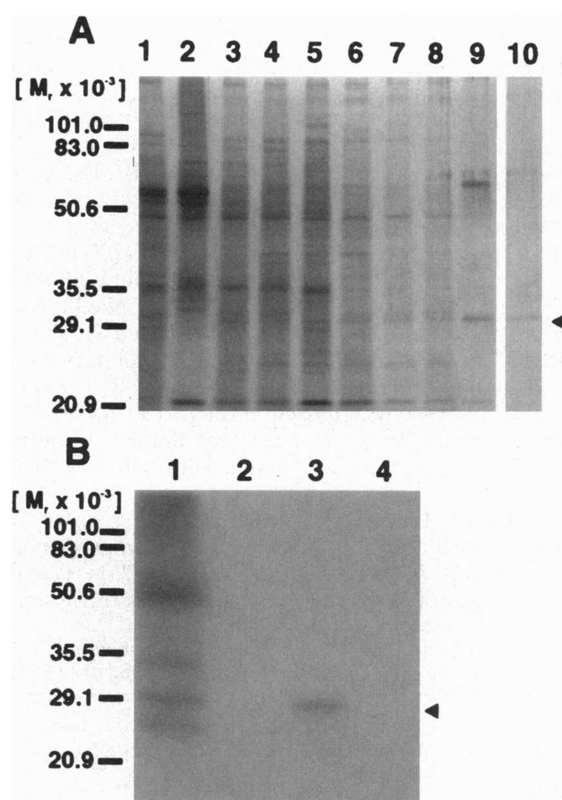

FIG. 1. Purification and specific immunoprecipitation of the sigma$_1$-binding protein. (*A*) SDS/gel analysis of purification fractions. The following fractions were subjected to reducing SDS/15% (wt/vol) PAGE: solubilized microsomes (4 μg of protein, lane 1), Q-Sepharose eluate (4 μg of protein, lane 2), hydroxyapatite eluate (3 μg of protein, lane 3), SP-Sepharose flow-through (3 μg of protein, lane 4), lysine-Sepharose eluate (3 μg of protein, lane 5), heparin-Sepharose flow-through (1.8 μg of protein, lane 6), spermine-agarose flow-through (0.8 μg of protein, lane 7), ADP-agarose flow-through (0.8 μg of protein, lane 8), thiopropyl-Sepharose eluate (1 μg of protein, lane 9), and Green-agarose eluate (0.1 μg of protein, lane 10). The gel was stained with silver. The arrow indicates the migration of the sigma$_1$-binding site. (*B*) Specific immunoprecipitation of the (−)[^{3}H]azidopamil photoaffinity-labeled sigma$_1$-binding site with anti-PBP45. Microsomal proteins were photolabeled with (−)[^{3}H]azidopamil (ligand concentration, 21 nM; protein concentration, 0.5 mg/ml) and immunoprecipitated as described, employing 35 μl of preimmune (lane 2) or anti-PBP45 serum in the absence (lane 3) or presence (lane 4) of 1 μM antigenic peptide. Lane 1 shows the migration of (−)[^{3}H]azidopamil-photolabeled liver microsomes. The fluorogram after 10 days of exposure is shown.

Table 2. Purification of the sigma$_1$-binding site from guinea pig liver microsomes

Fraction	Protein, mg	Recovery, %	(+)[^{3}H]Pentazocine binding, pmol	Recovery, %	Density, pmol/mg	Purification, fold
Microsomal extract	1,680.2	100	5210	100.0	3.1	1
Q-Sepharose	546.9	32.6	3575	57.7	6.5	2
Hydroxyapatite	85.5	5.1	1385	22.3	16.2	5
SP-Sepharose	54.9	3.3	1415	22.8	25.8	8
Lysine-Sepharose	15.1	0.9	845	13.6	55.8	18
Heparin-Sepharose	3.8	0.2	675	10.9	178.4	58
Spermine-agarose	2.2	0.1	560	9.0	259.8	84
ADP-agarose	2.0	0.1	580	9.4	298.0	96
Thiopropyl-Sepharose	0.2	0.01	205	3.3	1127.7	364

Purification was performed as described. Data shown were obtained from a typical experiment. (+)[^{3}H]Pentazocine binding activity was measured in duplicates at a ligand concentration of 0.4 nM by incubation for 12 h at 22°C in the presence of 0.3 mg of phosphatidylcholine per ml. Nonspecific binding was measured in the presence of 1 μM (±)-pentazocine. Protein concentrations were determined as described by Bradford (32) using BSA as a standard.

(+)[^{3}H]pentazocine binding in liver is reversible, saturable, and of high affinity, and it exhibits all pharmacological properties described for sigma$_1$-binding sites in mammalian brain.

To ensure that no other proteins are involved in the formation of the high-affinity (+)[^{3}H]pentazocine-binding site, we determined its target size. The analysis of (+)[^{3}H]pentazocine binding at single ligand concentrations revealed a simple exponential inactivation curve. From this curve a molecular mass of 34 ± 7 kDa (n = 9) was obtained. Saturation studies with (+)[^{3}H]pentazocine revealed an increase of the K_d values upon irradiation (data not shown). We corrected for this K_d increase as described (35) and calculated a molecular mass of the binding protein of 24 ± 2 kDa (n = 4).

Purification and Partial Amino Acid Sequencing of the Sigma$_1$-Binding Site from Guinea Pig Liver Microsomes. With (+)[^{3}H]pentazocine as a reversible ligand, we followed sigma$_1$-binding activity after solubilization in 1% (wt/vol) digitonin throughout a purification protocol employing eight chromatographic steps (Fig. 1*A*). Low binding recoveries were obtained from some resins to which crude or partially purified fractions were adsorbed. This was minimized by adding 0.3 mg of phosphatidylcholine per ml to the binding assay, facilitating further purification with reasonable recoveries (see Table 1). In the purified preparation, the (+)[^{3}H]pentazocine binding activity was enriched 364-fold [K_d = 1.0 ± 0.2 nM; B_{max} = 2.0 ± 0.1 nmol per mg of protein (n = 3); Table 2) as compared with the microsomal starting material. (+)[^{3}H]Pentazocine binding to the purified preparation showed essentially unchanged affinity for a variety of sigma-ligands (e.g., haloperidol, pentazocine, ditolylguanidine, ifenprodil, (+)- and (−)-SKF10,047, and progesterone). To obtain a more precise estimate of the molecular mass of the binding protein the thiopropyl-Sepharose eluate was separated on SDS/polyacrylamide gels of different acrylamide concentration. The relative migration of the purified protein was analyzed as described (28), yielding an apparent molecular mass of 29.7 ± 0.3 kDa (n = 2). The migration in SDS/PAGE was not affected by the previous reduction of disulfide bonds.

The ligand-binding polypeptide was identified by (−)[^{3}H]azidopamil photolabeling (data not shown) and electroeluted after preparative SDS/PAGE. Partial amino acid sequence information was obtained from tryptic fragments by gas-phase sequencing. To verify that the amino acid sequence of the tryptic peptide SEVFYPGETVVHGPGEATAVEWG originated from the sigma$_1$-binding site, we raised sequence-directed antibodies against a corresponding synthetic peptide (anti-PBP45). These antibodies specifically immunoprecipitated the (−)[^{3}H]azidopamil photolabeled sigma$_1$-binding site (Fig. 1*B*).

Molecular Cloning of the cDNA. Degenerate PCR primers were designed on the basis of the above sequence and used to amplify a 59-bp fragment. A specific oligonucleotide in conjunction with a poly(T) 3′-oligonucleotide allowed the amplification of a 1035-bp cDNA, which we used to screen a random-primed guinea pig liver cDNA λGT10 library. The open reading frame of a 1857-bp length clone (λGP8) coded for a protein with 223 aa and a calculated molecular mass of 25,314 Da. This was in agreement with the mass determined by radiation inactivation (24 ± 2 kDa) and SDS/PAGE (29.7 ± 0.3 kDa). The amino acid sequence contained the sequences of all tryptic digestion products. Two arginine amino acid residues (underlined) were found in the N terminus (MQWAVG<u>RR</u>). This putative amino acid sequence motif could act as an endoplasmic reticulum retention signal (45), in agreement with the previously reported subcellular localization of the sigma$_1$-binding site (27). A FASTA search revealed

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GP                     MQWAVGRRWLWVALFLAAVAVLTQIVWLWLGTQNFVFQREEIAQLARQYAGLDHELAFSKLIVELRRLHPVHV
MG                MSSPISGFLRF.AVLLAV.SPLVYLAEQRLESFYVFDHKHLHELSTQA...HGNNTRAIVGHIVDELRARPETTKYI---
SC                     MKFFPLLLLIGVVGYIMNVLFTTWLPTNYMFDPKTLNEICNSV.SKHNAAEGLSTED.LQDVRDALASHYGDEYI
UM MASHRPRSNKAANGASTSPKRSWII.SAALVGFCALI..LDSIRSSFYIFDHKAIYKIASTAV.NHPGNATAIFDDVLDNLRADPKLAPYINKN

GP LPDEELQWVFVNAGGWMGAMCLLHASLSEYVLLFGTALGSPRHSGRYWAEISDTIISGTFHQWREGTTKSEVFYPGETVVHGPGEATAVEW-GP
MG --SVQEE...N....A..G.Y.I...VT..L.I....I.TEG.T..HT.DDYFN.LT.EQWAYVP.EYEP..YPA.SVHHLRR.TVKQYKMPE-
SC NRYVKEE...N....A..Q.I.....V...L......V.TEG.T.VHF.DDYF..LH..QIAALPYA.EA..YT..M.HHLKK.Y.KQYSMP.-
UM HFSD.SE.M.N....A..S.F.I...VT..L.F...PV.TEG.T..HT.DDYFN.LT.NQYAFPA.AL.A.HYPA.SVHHLRR.TVKQYMMPED

GP NTWMVEYGRGVIPSTLGFALADTVFSTQDFLTLFYTLRVYARALQLELTTYLFGQDP
MG GCFAL..A..W..PM.F.GF..GLS..L..P..WQ.TV.TG.EMLGN.MLGK.
SC GSFAL.LAQ.W..CM.P.GFL..FS..L.LY..YR.VYLT..DMGKN.LQNKKF
UM GC.AL.LAQ.W..PM.P.G...VLS..L.LP.FGI.VWIT..EMVGN.LIGK.
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FIG. 2. Alignment of the deduced amino acid sequences of the sigma$_1$-binding protein from guinea pig (GP) and the ERG2 gene products from *M. grisea* (MG; ref. 46), *S. cerevisiae* (SC; ref. 48), and *U. maydis* (UM; ref. 46). Amino acid residues differing from the guinea pig sequence are given, whereas identical residues are omitted. Gaps are indicated by the lines. The sequences of the four tryptic peptides determined by gas-phase sequencing are overlined, and the peptide used for antibody generation is marked in bold. A potential transmembrane segment predicted by the hydrophobicity analysis according to Kyte and Doolittle (49) with a window of 19 aa is shaded.

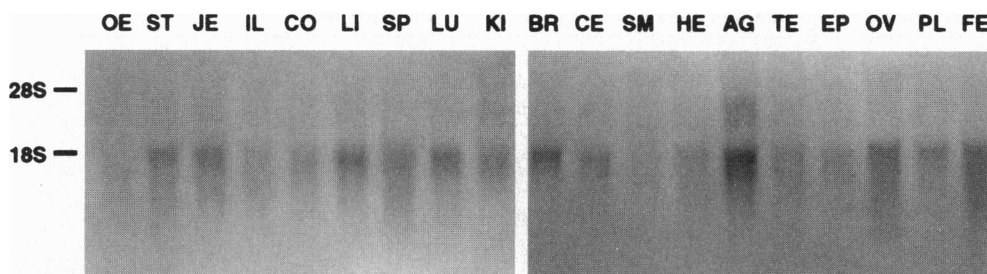

FIG. 3. Northern blot analysis of total RNA from guinea pig tissues. RNA preparation, electrophoretic separation, blotting, and hybridization with a 1800-bp λGP8 probe were performed as described (29). Total RNA (10 μg) from the following tissues were analyzed: oesophagus (OE), stomach (ST), jejunum (JE), ileum (IL), colon (CO), liver (LI), spleen (SP), lung (LU), kidney (KI), brain (BR), cerebellum (CE), skeletal muscle (SM), heart (HE), adrenal gland (AG), testis (TE), epididymis (EP), ovary (OV), placenta (PL), and fetus (FE) of 20-day gestation.

amino acid sequence homology with ERG2, a fungal gene encoding sterol isomerases in *Magnaporthe grisea*, *S. cerevisiae* and *Ustilago maydis* (46–48). In a 218-aa overlap, these sequences were 30.3% identical and 66.4% similar with the sigma$_1$-binding site (Fig. 2). This suggests that the protein forming the sigma$_1$-binding site represents the mammalian counterpart of fungal sterol C_8-C_7 isomerase. This microsomal enzyme, which was not yet purified, shifts the C_8 double bond of zymosterol to position C_7, an essential step in sterol synthesis (50–54). Hydrophobicity plots according to ref. 49, with a window of 19 aa, predicted one putative transmembrane segment at the N terminus for the sterol isomerases as well as for the sigma$_1$-binding protein (data not shown). This segment may anchor the protein in the membrane, whereas two shorter C-terminal hydrophobic amino acid stretches could be involved in sterol binding (46).

Northern blot analysis of total RNAs isolated from different guinea pig tissues showed that they all expressed an ≈2000-nt transcript. The tissue distribution of the mRNA was in agreement with its proposed participation in sterol synthesis (55). Although the mRNA was present in all tissues analyzed, the highest densities were found in liver, kidney, and steroid-producing tissues (Fig. 3) such as placenta, ovary, and adrenal gland. The high density of the mRNA in brain could be due to the high cholesterol demand of the developing brain in young animals (56, 57).

Heterologous Expression of the cDNA in *S. cerevisiae*. The sigma$_1$-binding site is defined by its ability to bind drugs such as haloperidol, (+)-pentazocine, (+)-3-propyl-3-(3-hydroxyphenyl)piperidine, and ditolylguanidine with high affinity and progesterone with moderate affinity. To establish that the cloned cDNA encoded a protein forming the sigma$_1$-binding site, we functionally expressed the cDNA in *S. cerevisiae*. Cells transformed with the open reading frame or the open reading frame tagged either with a 6xHIS or a 9E10 c-*myc* epitope expressed high-affinity binding sites for (+)[^{3}H]pentazocine (Fig. 4*A*) and [^{3}H]ditolylguanidine (data not shown) as well as anti-PBP45 immunoreactivity (Fig. 4*B*). No binding activity was found in membranes from mock-transformed cells. The (+)[^{3}H]pentazocine binding properties of the 6xHIS-tagged sigma$_1$-binding site were essentially identical with the properties of the native binding site in guinea pig liver and brain (Table 1). This unequivocally demonstrates that the cloned cDNA encodes a protein that forms the high-affinity (+)[^{3}H]pentazocine-binding domain characteristic for mammalian sigma$_1$-binding sites.

Consequences for Sigma-Ligand Research. The sigma-receptor was originally postulated by Martin *et al.* (1) as an opiate receptor subtype mediating psychomimetic effects of SKF10,047. Later the term sigma-receptor was assigned to high-affinity binding sites in brain that interact with so-called sigma-ligands with high affinity and a characteristic pharmacological profile. We purified and cloned the sigma$_1$-subtype in an attempt to clarify its functional role. Our work demonstrates that the sigma$_1$-binding site, defined as a high-affinity, haloperidol- and progesterone-sensitive binding domain for (+)-benzomorphans such as pentazocine, is localized on a protein with similarities to the fungal gene product ERG2, which encodes a sterol isomerase. For the mammalian isomerase a cell-free functional assay has been described (50, 51, 58), but no standard procedures are currently available to measure its activity. In contrast, in yeast, isomerase activity can be detected spectrophotometrically (40) because ergosterol in yeast contains an UV-absorbing conjugated ring system. We therefore tested whether the sigma-ligand-binding protein reverts the phenotype of an ERG2 mutant strain of *S. cerevisiae* devoid

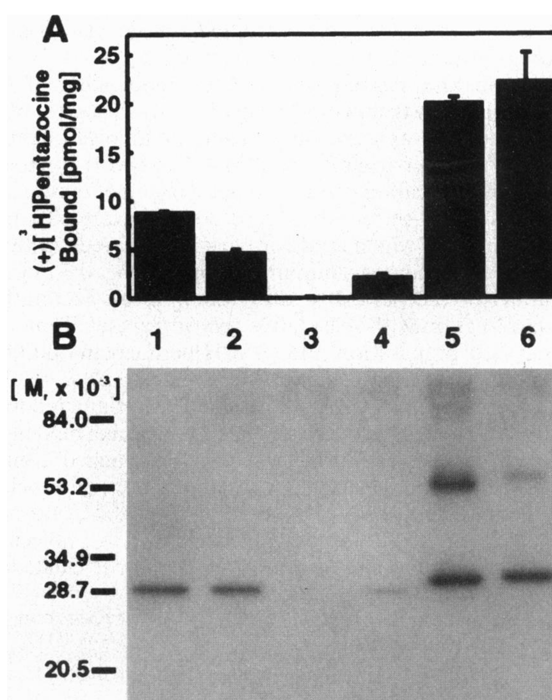

FIG. 4. Heterologous expression of the cloned cDNA in *S. cerevisiae*. Transformation of *S. cerevisiae*, cultivation, and membrane preparation were performed as described (29). (*A*) (+)[^{3}H]Pentazocine binding to membranes from guinea pig liver (column 1) and brain (column 2) and *S. cerevisiae* cells transformed with the vector alone (column 3), λGP8-ORF (column 4), 6xHIS-λGP8-ORF (column 5), and 9E10 c-*myc*-λGP8-ORF (column 6) was assayed at a ligand concentration of 0.5 nM at 22°C at pH 8.3. (*B*) For immunoblotting, 5 μg and 1 μg of microsomal guinea pig and yeast protein, respectively, were subjected to SDS/PAGE and immunostaining as described (28). The migration of the molecular weight standards and of the sigma$_1$-binding protein (arrow) are given on the left and right, respectively. 6xHIS as well as 9E10 c-*myc* tagging decreased electrophoretic mobility and lead to partial dimerization (stained 55-kDa band). No immunostaining was observed in the presence of 1 μM of synthetic peptide (data not shown).

of endogenous isomerase activity [strain WA0 *(a his7-2 leu2-3,112 ura3-52 erg2-3)*; ref. 47]. In contrast to the overexpressed ERG2, the cloned cDNA did not normalize the spectrophotometric profile of nonsaponifiable sterols extracted from transformed cells, indicating that yeast-like isomerase activity was absent ($n = 2$; data not shown). Either the mammalian isomerase cannot use fecosterol instead of zymosterol as a substrate, or the fungal enzymes downstream, such as the C_{24}-methylase (ERG6) or the C_5-desaturase (ERG3), do not recognize the product generated by the mammalian isomerase, which removes the 7-β-hydrogen, whereas in fungi the 7-α-hydrogen is lost upon isomerization (58, 59). Although we were not yet able to reveal the biological function of the cloned cDNA, the affinity of sigma$_1$-binding sites for steroids, such as progesterone [K_i for (+)[^{3}H]pentazocine binding, 260–570 nM; see Table 1] indicates that this protein carries a sterol/steroid-binding domain obligatory for a protein involved in sterol synthesis. The existence of a lipohilic sterol/steroid-binding site also explains the surprising ability of our 25.3-kDa protein to bind so many drugs from distinct pharmacological classes with high affinity. Sterol isomerase inhibitors and presumably also sigma-ligands mimic the unstable carbocationic isomerization intermediate (59, 60). Many transition state analogues are slow-binding inhibitors (61) in accordance with the slow association of (+)[^{3}H]pentazocine binding.

Further functional studies will be necessary to definitely establish the biological role of this protein. Future investigations relating drug effects to their action at sigma$_1$-binding sites will have to explain functional data on the basis of the structure and presumed function of the novel mammalian protein presented here. This will pave the way to unequivocally establishing the pharmacological significance of sigma$_1$-binding sites.

We thank Drs. Maria L. Garcia and Gregory J. Kaczorowski (Merck Research Laboratories, Rahway, NJ) for providing protein-sequencing facilities; Dr. Manfred Grabner for advice and comments; Dr. P. Benveniste (Strasbourg, France) for discussion; Drs. Michael Ahlijanian (Pfizer, Groton, CN) and Sigi Schwartz (Innsbruck) for helpful comments on our manuscript; Dr. Martin Bard (Purdue University, Indianapolis) for providing yeast strain WA0 and the ERG2 cDNA; and Marlies Holzer for expert technical assistance. This work was supported by a Boehringer-Ingelheim fellowship (F.F.M.); the Dr. Legerlotz Foundation (J.S.); grants from the Österreichische Nationalbank (H.G.); and the Fonds zur Förderung der wissenschaftlichen Forschung, Austria (Grant S6601 to H.G.; Grant P9351 to J.S.). This work is part of the thesis of A.F.

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