Expression cloning of cDNA encoding a seven-helix receptor from human placenta with affinity for opioid ligands

GUO-XI XIE*†, ATSUSHI MIYAJIMA*‡, AND AVRAM GOLDBEIN§

*DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304; and §Stanford University, Stanford, CA 94305

Contributed by Avram Goldstein, December 27, 1991

ABSTRACT Here we report the expression cloning of a seven-helix guanine nucleotide-binding protein (G-protein)-coupled receptor family. Ligand binding reveals a binding site with stereochemical selectivity. The deduced transmembrane domain is 93% identical to the homologous region of the human neuromedin K (neurokinin B) receptor, but the N-terminal and C-terminal sequences have many dissimilarities. The expressed receptor binds opioid ligands but not tachykinins; and under the same conditions, a cloned rat neuromedin K receptor binds tachykinins but not opioids.

Saturable stereospecific binding of an opiate ligand was first demonstrated by one of us in 1971 (1), and since then multiple opioid receptors have been characterized (2–5), but cloning has not yet been achieved to our knowledge. The cloning of an opioid-binding protein, OBCAM (6), has been reported; but as it has no transmembrane domain, OBCAM seems not to be a receptor. For expression cloning of cDNA encoding the dynorphin (Dyn) (κ opioid) receptor (7) by ligand binding, we turned to transient transfection of COS-7 cells and affinity enrichment (panning), followed by successive dilutions of plasmid pools. The cDNA library was derived from human placenta, a rich source of κ receptors (8).

MATERIALS AND METHODS

Vector and Library Construction. We used the pME18S vector (9), a high-copy-number small-vector [3.4 kilobases (kb)] plasmid with a strong promoter (10), suitable for constructing size-selected unidirectional cDNA libraries and for mammalian expression. Total RNA was isolated from fresh human placenta by guanidinium isothiocyanate extraction followed by centrifugation in cesium chloride (11), and poly(A)* RNA was purified by using an oligo(dT)-cellulose column (Pharmacia). Synthesis of cDNA (12) was with a Promega kit. First-strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase with oligo(dT)-Not I primer–adapter [oligo(dT)$_{12}$ containing the Not I site on its 5' end]. Second-strand cDNA was synthesized by using Escherichia coli DNA polymerase I and RNase H. After treatment with T4 DNA polymerase to blunt the ends, the double-stranded cDNA was ligated with Bst XI linker (Invitrogen) and T4 ligase at 14°C for 24 hr. After a treatment with Not I to create sticky ends, the cDNA was fractionated on 1% agarose gel by electrophoresis, and fractions > 1.5 kb were electroeluted to DE-81 ion-exchange paper (Whatman) and eluted with 1 M NaCl. After precipitation with ethanol, the cDNA was washed and unidirectionally inserted by T4 ligase into the BsrXI–Not I sites of pME18S. The resulting cDNA library had 1.4 × 10$^6$ independent colonies after transformation of DH5α competent E. coli by electroporation.

Affinity Enrichment (Panning). In preparation for expression cloning, we had developed a set of modified (13) and chimeric (14) opioid peptides, the latter based on the structure of Dyn-32. Dyn-32 is the 17-residue DynA linked at its C terminus through Lys-Arg to DynB (15). We had also raised a monoclonal antibody (mAb), 17. M, that recognized the C-terminal sequence of Dyn-32 (15), leaving the opioid-active κ-selective N-terminal domain free to interact with cell-surface receptors. These reagents were used for panning. COS-7 cells were transfected (16) as follows. Exponentially growing cells were plated (5 × 10$^5$ per 10-cm dish) in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS). After 24 hr at 37°C in a 5% CO$_2$/95% air incubator, cells were washed twice with Iscove’s modified Dulbecco’s medium (IMDM), which contains l-glutamine and 25 mM Hepes (pH 7.4). Plasmid DNA [10 μg, purified by CsCl gradient centrifugation (11)], DEAE-dextran (M, 500,000, 0.2 mg/ml; Pharmacia), and chloroquine (100 μM) were mixed in 4 ml of IMDM and added to the dish, which was returned to the incubator for 5 hr. The cells were washed with IMDM and then with DMEM containing 5% FCS and were cultured in 10 ml of DMEM with 10% FCS for 3 days in the incubator.

The panning procedure itself was modified from Seed and Stutte (17). Petri dishes (60-mm diameter, FALCON 1007; Becton Dickinson) were coated with mAb 17. M (15) (30 μg per dish in 3 ml of 50 mM Tris-HCl buffer, pH 9.5) for 2 hr and then washed three times with 0.15 M NaCl. Phosphate-buffered saline [PBS; 3 ml containing 0.1% bovine serum albumin (BSA)] was added, and the dishes were incubated at 4°C overnight. Transfected cells (see above) were detached with PBS (GIBCO, without Ca$^{2+}$ and Mg$^{2+}$) containing 0.5 mM EDTA and 0.02% NaN$_3$. After washing the cells twice with Krebs-Hepes buffer (KHB; 118 mM NaCl/4.8 mM KCl/2.5 mM CaCl$_2$/1.2 mM MgCl$_2$/25 mM Hepes, pH 7.4), cells from two dishes were suspended in 1 ml of KHB containing 0.1% BSA and 100 nM Dyn-32 and were incubated 1.5 hr at room temperature and then 30 min on ice. After three washes to remove free Dyn-32, cells were resuspended in 1 ml of PBS containing 0.5 mM EDTA and 0.1% BSA and plated on the antibody-coated dishes. After 2 hr at 37°C, the colonies were fixed with 3% formaldehyde.

Abbreviations: Brem, bremazocine; Dyn, dynorphin; DynA and DynB, Dyns A and B; mAb, monoclonal antibody; hNK and rNK, human and rat neuromedin K receptors; NK, neuromedin K; SP, substance P; SK, substance K; G protein, guanine nucleotide-binding protein; DAGO, [D-Ala$^2$, N-MePhe$^5$, Gly$^b$-O�]enkephalin; DPDPE, [D-Pen$^2$, D-Pen$^5$]enkephalin in which Pen is penicillamine; G protein, guanine nucleotide-binding protein.

To whom requests for the hKIR clone should be addressed at:
Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109.

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room temperature, the dishes were washed gently three times with 3-ml portions of PBS. Then cells remaining on the dishes were lysed, and plasmid DNA was recovered by the Hirt method (18) and amplified in E. coli to obtain material for the next cycle of panning.

**Ligand Binding.** Binding assays were performed with whole COS-7 cells; membrane preparations gave similar results. As positive controls we included the H-187 line of small-cell lung cancer cells (kindly furnished by J. D. Minna, National Cancer Institute), which express about $5 \times 10^5 \kappa$ receptors per cell. As negative controls we used mock-transfected (plasmid alone, no cDNA insert) and untransfected cells.

Transfected cells were harvested in PBS (without Ca$^{2+}$ and Mg$^{2+}$) containing 0.5 mM EDTA and 0.01% NaN$_3$, then washed three times with KHB, and resuspended in KHB (10$^6$ cells per ml). Binding assays were performed with 10$^6$ cells in 2 ml of KHB containing radioligands and competitors. [3H]Bremazocine ([3H]Brem; 37.0 Ci/mmol, NEN; 1 Ci = 37 GBq), a high-affinity opioid ligand with modest selectivity for $\kappa$ sites, was used as radioligand. Assay tubes were incubated 2 hr at room temperature and then on ice for 5 min and were centrifuged (250 × g at 2°C for 5 min). Free radioligand concentrations (>80% of total) were measured in supernatants. Cell pellets were washed twice with ice-cold KHB and transferred to fresh tubes; radioactivity was determined in scintillation solution (Cytoscint, ICN Biochemicals) or in the $\gamma$ counter.

For competition studies [3H]Brem was used at about $2 \times 10^8$ cpm per tube (2.5 nM). Specific binding was defined as the reduction of bound radioligand by 4$\mu$M U-50488 (19) (see Fig. 1b). The following unlabeled opioid ligands were tested as competitors: DynA-(1-13), des-Tyr$^5$-DynA-(1-13), [d-Ala$^2$, N-MePhe$^4$, Gly$^9$-]enkephalin (DAGO), and [d-Pen$^5$, d-Pen$^6$]enkephalin (DPDPE) in which Pen is penicillamine (Peninsula Laboratories); U-50488, U-63939, and U-63940 (Upjohn); levorphanol and dextrophan (Roche); and naloxone and naltrexone (Endo Laboratories, New York). The following tachykinin radioligands were used: [3H]oleptodisin (38.2 Ci/mmol, NEN); radiodinated substance K, [2-($^{35}$S)iodohistidyl]$^5$SK (2,000 Ci/mmol, Amersham); and tritiated substance P [2-propyl-3,4-$^{3}$H$_2$]-Pen$^2$, Met(O$_2$)$^{33}$SP in which Sar is sarcosine (32.5 Ci/mmol, NEN). Unlabeled neuromedin K (NK), SP, and SK (Peninsula) were tested as competitors. Specific binding was determined with 500 nM unlabeled NK, SP, or SK as appropriate.

**Sequence Analysis.** Both strands of cloned cDNA were sequenced (20) by using both double-stranded (pME18S-cDNA) and single-stranded (M13mp19, Boehringer Mannheim) templates with Sequenase (United States Biochemicals) and Taq polymerase as required. For some G+C-rich regions, dITP and 7-deaza-dGTP were used. Sequences were confirmed in both directions by multiple redundant reactions and gel loadings. Especially in G+C-rich regions we found some consistent disagreements between 5'-to-3' and 3'-to-5' sequencing gels, usually with a missing nucleotide in one sequence or the other; in these cases we assumed that the extra nucleotide was correct. When single- and double-strand readings disagreed, we accepted the former. DNA database search was by FASTDB (IntelliGenetics); DNA and deduced protein sequences were analyzed by the Wisconsin and Intelligenetics programs.

**RESULTS**

**Isolation of cDNA.** At the fourth panning cycle, 80% of the cells could be prevented from attaching by competition with the $\kappa$-opioid receptor-selective ary lacetamide ligand U-50488.

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*The sequence reported in this paper has been deposited in the GenBank database (accession no. M84605).*

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**Fig. 1.** Binding isotherm and competition. Data are means ± SEM for multiple experiments, with triplicate determinations at each point in each experiment. (a) [3H]Brem binding isotherm. (b) Competition by U-50488 (○) and DynA-(1-13) (□); triplicate determinations in one experiment).

**Fig. 2.** Ligand competition profiles. Competitors were at 500 nM unless otherwise indicated in parentheses. Solid bars indicate reduction of [3H]Brem binding by competitor, expressed as percent of specific binding. n, Number of experiments.
saturation with a $K_d$ of 87 nM (Fig. 1a). Competition curves for both U-50488 and DynA-(1-13) yielded IC$_{50}$ values (here equivalent to $K_d$) of about 100 nM (Fig. 1b). The maximum reduction in binding by competition was approximately equal to the increment in binding due to transfection.

Competition results obtained with various ligands are shown in Fig. 2. U-50488 gave near-maximal competition, and DynA-(1-13) competed about as well, but des-Tyr-

DynA-(1-13) did not compete at all. Levorphanol (a $\mu$-opioid receptor-selective morphinan) competed, but its (+)-enantiomer dextrorphan (21) did not. Two other $\mu$-selective morphinans, naloxone and naltrexone, competed poorly.

Surprisingly, the peptides DAGO (19) ($\delta$-selective) and DPDP (19) ($\delta$-selective) competed about as well as U-50488. The inability of the site to distinguish $\mu$-selective ligands was also evident from the fact that

**Figure 3.** (a) Nucleotide and deduced amino acid sequence (in single-letter code) of hK1R and comparison with deduced amino acid sequence of hNK3R (22). The sequence of hNK3R is shown under that of hK1R. Nucleotides are numbered on the left and amino acids on the right. Possible N-glycosylation sites (+) and stop codons (>) are marked. Alignment was by the Wisconsin GAP program, with gaps inserted to maximize matches. Colons indicate identities. The following sequence in hNK3R precedes the start of hK1R: MATLPAAETWIDGGGG. Gaps: +, gap in hK1R after amino acid residue 321 with insert GWLQLDDLQAG in hNK3R; #, gap in hK1R after residue 339 with insert VA in hNK3R; ***, three-residue gap in hNK3 opposite residues 41-43 of hK1R. Putative hydrophobic transmembrane domains TM-1 through TM-7 are according to ref. 22. (b) Hydrophobic plot as described by Kyte and Doolittle (23), for hK1R showing hydrophobic segments upward and residue number on the x-axis.
Sequence Analysis. Fig. 3a shows the nucleotide and deduced amino acid sequence of hK1R cDNA and also of human NK receptor (hNKR; see below). In hK1R the insert of 4839 base pairs is followed by a poly(A) tract of 22 nucleotides. A long open reading frame after the first ATG encodes a protein of 440 residues (including the methionine) and calculated Mr of 49,422. The initial ATG (in CCCATGG) meets the Kozak consensus criterion (ACCATGG) (24) only moderately well; as the insert has no upstream in-frame stop codon, additional 5' coding sequence cannot be excluded.

Fig. 3b shows the hydrophobicity analysis according to Kyte and Doolittle (23). No signal sequence is present. Seven hydrophobic segments are evident, each long enough to form a membrane-spanning α-helix. These are placed in Fig. 3a according to the assignments for the tachykinin receptors (22) (see below), but additional flanking hydrophobic residues could have been included in all but transmembrane domains TM-3 and TM-4. In the N-terminal domain are two potential N-glycosylation sites, at amino acid residues 7 and 24. Cysteine residues occur in the first and second extracellular loops. In the C-terminal cytoplasmic tail, 27% of the residues are serine or threonine, suggesting possible regulation by protein kinases.

A search of the nucleic acid data bases revealed that the greatest similarity of hK1R was to receptors of the 7-helix family and especially to the rat NK receptor (rNKR), with 79% identity of the deduced protein sequences (three gaps). S. Nakanishi (Kyoto University) provided the rat clone (rNKR-CDM8) (25), which was expressed in COS-7 cells.

Untransfected cells bound radiolabeled eledoisin hardly at all, whereas cells transfected with rNKR (but not hK1R) bound it very well (Table 1). Radiolabeled SP and SK ligands bound relatively poorly (not shown). NK was the most potent tachykinin tested as competitor (K, 5 nM), reducing binding by 95% to the mock level. These results are in general agreement with the report of Nakanishi's group (25). These cells showed no specific binding of [3H]Brem, nor did the k-selective opioid ligand U-50488 compete with [3H]eledoisin. In contrast, hK1R-transfected cells in the same experiments bound [3H]Brem specifically but did not bind any of the tachykinin radioligands, nor did the tachykinins compete against [3H]Brem binding. Because of the considerable nonspecific binding of [3H]Brem, U-50488 reduced total binding by only 19%, but this represented 64% reduction of specific binding or of the incremental binding caused by transfection.

Table 1. Ligand binding to hK1R and rNKR

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>n</th>
<th>Competitor (500 nM)</th>
<th>cpm, mean ± SEM</th>
<th>Competition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Eled</td>
<td>4</td>
<td>Untransfected</td>
<td>44 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Control</td>
<td>3688 ± 86</td>
<td>4341 ± 45</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>U-50488</td>
<td>4256 ± 77</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>NK</td>
<td>213 ± 20</td>
<td>301 ± 30</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>SP</td>
<td>2798 ± 37</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>SK</td>
<td>3084 ± 43</td>
<td>17</td>
</tr>
<tr>
<td>[3H]Brem</td>
<td>28</td>
<td>Untransfected</td>
<td>3776 ± 270</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Control</td>
<td>3682 ± 392</td>
<td>5033 ± 96</td>
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<tr>
<td></td>
<td>8</td>
<td>U-50488</td>
<td>3962 ± 430</td>
<td>4949 ± 357</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NK</td>
<td>3515 ± 280</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>SP</td>
<td>3882 ± 654</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>SK</td>
<td>3757 ± 32</td>
<td>4992 ± 107</td>
</tr>
</tbody>
</table>

All data are total binding. "Control" is the binding of radioligand alone to transfected cells. Competition is the percent reduction of control binding. Columns a are independent transfections (number in column n) carried out over a period of weeks; columns b are independent transfections (second number in column n) carried out in a single day. To show clearly the differences in nonspecific binding between the two expressed receptors, raw counts are given here rather than specific binding data. [3H]Eled, [3H]eledoisin.

DISCUSSION

That the protein encoded by hK1R is an opioid receptor is supported by the following four considerations. (i) Opioid receptors bind both peptide and nonpeptide ligands with stringent structural requirements. The pattern of binding here is classically opioid in that both opioid nitrogenous bases (e.g., Brem, U-50488 and other arylacetamides, and levorphanol) and opioid peptides (e.g., Dyn-A(1-13), DAGO, and DPDPE) are bound. (ii) N-terminal tyrosine is a nearly
universal requirement for recognition of opioid peptides by opioid receptors. Therefore, it is significant that whereas DynA(1–13) binds, its des-Tyr derivative does not. (iii) Stereoselectivity is a feature of all known opioid receptors. The site expressed here is stereoselective, as revealed by the contrast between levorphanol and its enantiomer dextrophan. (iv) Binding and functional behavior of opioid receptors depend on coupling to a guanine nucleotide-binding protein (G protein), probably Gq or Gs. It is significant, therefore, that the hydropathy plot here reveals a seven-helix structure, typical of receptors coupled to G proteins.

As a κ-receptor-selective peptide ligand was used for panning, and as human placenta (source of the cDNA library) contains chiefly κ receptors, we had expected to find typical κ-receptor affinity (low nanomolar range) and also a preference (by at least 1 order of magnitude) for κ-selective over μ- and δ-selective ligands. Thus, the observed affinity (2 orders of magnitude lower) and the poor selectivity of the expressed receptor were unexpected. There are five possible explanations. (i) As there is no stop codon upstream of the first ATG, a 5′ coding sequence that makes a major contribution to affinity and selectivity could be missing. (ii) The correct G protein could be missing from the COS-7 cells, as found recently with a secretin receptor, which also belongs to the seven-helix family (27). As the binding affinity in our experiments was unchanged in washed membrane preparations, a high cytosolic GTP concentration (well known to reduce binding affinity) (26) could not be responsible. (iii) An accessory protein other than a G-protein could be required for high-affinity binding and selectivity. More than a single polypeptide is sometimes required for high-affinity binding [e.g., interleukin 2 receptor (28)], but this has not been found for any G-protein-coupled receptor. (iv) A posttranslational modification could be needed for high-affinity binding but not carried out by COS-7 cells. It is noteworthy that there are two potential glycosylation sites in the extracellular N-terminal domain of the deduced sequence. (v) This opioid receptor is actually of a novel type, which does not discriminate among the classical type-selective ligands. A nonselective opioid receptor has been described (29), but it has very high affinity for fentanyl, and δ opioids.

That a receptor with opioid binding properties should be a member of the tachykinin receptor family is interesting from an evolutionary standpoint. The similarity to hNK1R poses the question of what residues are responsible for ligand recognition. S. Nakanoishi kindly informed us in advance of publication of the deduced amino acid sequence of hNK1R (22). The comparison is shown in Fig. 3a. Overall, there is 81% sequence identity (with three gaps), but the distribution of identical and nonidentical residues is very uneven. Throughout the central transmembrane region the sequences are 93% identical (no gaps), and the cytoplasmic C-terminal tails are 74% identical (no gaps). The extracellular N-terminal domains differ greatly (39% identical, three gaps). Therefore, we suggest that the very different ligand binding specificities of NKR and hNK1R are determined in large part by the extracellular N-terminal domains, which are substantially different in the two receptors. Furthermore, hNK1R and rNK1R have three and four potential glycosylation sites, respectively, as compared with two in hK1R. This model is consistent with thyrotropin and luteinizing hormone receptors (30) as well as tachykinin receptors (22). That ligands of seven-helix receptors bind in a pocket in the membrane was deduced from mutagenesis studies with small nonpeptide ligands (31). For recognition of the extended pharmacophore of a peptide, the N-terminal extracellular domain of the receptor may be required. However, this hypothesis would not resolve the long-standing problem of how extended opioid peptides and compact opioid nonpeptides are recognized by the same binding site.

A final caveat is in order. Two segments of the coding sequence (beginning at nucleotides 18 and 585) contain alternative long open reading frames encoding 188 and 171 amino acids, respectively. Remote as the possibility may be in light of the multiple intron deletions in our sequencing strategy, it is nevertheless conceivable that one or two nucleotides were missed, shifting the reading frame and resulting in a completely different deduced protein sequence. Analogous cases are known (32, 33). However, the hydropathy plot for other reading frames of hK1R gives no indication of multiple transmembrane helices.

We thank Drs. K. Ariai and T. Yokota for helpful advice and guidance. Dr. K. Maruyama for providing PME18S. D. Robison for synthesis of oligonucleotides, Dr. R. C. Thompson for assistance with the NKR clone, Dr. J. D. Minna for the H-187 cell line, Dr. P. von Voigtlander for Upjohn arylacetamide ligands, and Dr. S. Nakanoishi for providing the rat NK1R clone and for information about the amino acid sequence of hNK1R. Drs. H. Akil, D. B. Goldstein, F. Lee, L. Stryer, J. A. Waitz, and S. J. Watson kindly commented on earlier drafts of the manuscript. DNAX Research Institute is supported by Schering-Plough Corporation.