Novel “Restoration of Function” Mutagenesis Strategy to Identify Amino Acids of the δ-Opioid Receptor Involved in Ligand Binding*

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A novel “restoration of function” mutagenesis strategy was developed to identify amino acid sequence combinations necessary to restore the ability to bind δ-selective ligands to an inactive δμ receptor chimera in which 10 amino acids of the third extracellular loop of the δ receptor were replaced by the corresponding amino acids from the μ receptor (δ/μ 291–300). This chimera binds a nonselective opioid ligand but is devoid of affinity for δ-selective ligands. A library of mutants was generated in which some of the 10 amino acids of the μ sequence of δ/μ 291–300 were randomly reverted to the corresponding δ amino acid. Using a ligand binding assay, we screened this library to select mutants with high affinity for δ-selective ligands. Sequence analysis of these revertants revealed that a leucine at position 300, a hydrophobic region (amino acids 295–300), and an arginine at position 291 of the human δ-μ opioid receptor were present in all revertants. Single and double point mutations were then introduced in δ/μ 291–300 to evaluate the contribution of the leucine 300 and arginine 291 residues for the binding of δ-selective ligands. An increased affinity for δ-selective ligands was observed when the tryptophan 300 (μ residue) of δ/μ 291–300 was reverted to a leucine (δ residue). Further site-directed mutagenesis experiments suggested that the presence of a tryptophan at position 300 may block the access of δ-selective ligands to their docking site.

The opioid receptors are widely distributed throughout the central nervous system and mediate the diverse effects of endogenous opioid peptides and opiate drugs (1). Pharmacological studies have defined at least three classes of opioid receptors, named δ, μ, and κ, that differ in their affinity for ligands and in their distribution in the nervous system (1–6).

The antinociception mediated by supraspinal opioid receptors is thought to act via the μ-opioid receptor subtype (7–10). The numerous side effects accompanying opioid treatment, including respiratory depression and addiction (11), are generally thought to be mediated by the stimulation of μ receptors. However, there is growing evidence suggesting that selective stimulation of the δ receptor may also mediate antinociception (12–19). The strongest indication of an involvement of δ-opioid receptors in supraspinal antinociception follows from studies with selective antagonists (14–16). These studies demonstrated that the antinociception produced by intracerebroventricular injection of morphine and [α-Ala²,MePhè⁴,Gly-ol⁵]-enkephalin (μ agonists) was antagonized by β-funaltrexamine and naloxone (μ antagonists) but not by ICI-174864 (δ antagonists). Conversely, the antinociception produced by intracerebroventricular injection of DPDPE (δ agonist) was antagonized by ICI-174864 but not by β-funaltrexamine and naloxonazine. Moreover, studies have shown that an antisense oligodeoxyribonucleotide to the cloned δ-opioid receptor given intrathecally lowers δ but not μ or κ spinal (20) and central (21) analgesia. These studies confirm, at the molecular level, traditional pharmacological studies implying distinct receptor mechanisms for δ, μ, and κ analgesia. The development of selective and potent δ-opioid agonists therefore presents the potential for the discovery of novel analgesic agents with reduced accompanying side effects.

The recent cloning of the genes encoding the opioid receptors showed that they are members of the seven transmembrane G protein-coupled receptor family (22–28). There is about 60% amino acid identity among the sequences of the three subtypes δ, μ, and κ. The highest sequence homology between the three opioid receptor subtypes resides in the transmembrane domains and the intracellular loops. Lower sequence homology is seen in the N and C termini, transmembrane domain 4, and extracellular loops 2 and 3. It is likely that some of these divergent regions contain elements responsible for the discrimination among these receptors by the subtype-selective opioid ligands. The construction of chimeric receptors is a powerful approach to investigate the structural basis for the subtype specificity of G protein-coupled receptor (29–34). Previous studies from our group (35) and others (36–40) have demonstrated, using chimeras, the importance of the third extracellular loop of the δ-opioid receptor for δ ligand selectivity.

Most mutagenesis experiments designed to analyze the structure and function of G protein-coupled receptor involve a strategy based on the loss of function. Thus, even in well controlled studies, the interpretation of these experiments is often difficult since a loss of function may result from various causes. A mutant receptor may lack affinity for a ligand because a critical residue of the binding pocket has been hit but also because the mutated receptor is unable to traffic efficiently to the cell surface or because the mutation induces protein misfolding, allosteric changes, or gross structural defect. Furthermore, the direct determination of G protein-coupled receptor three-dimensional structure is hampered by technical difficulties limiting their overexpression and purification in quantities that would permit crystallographic studies. Today, only relatively low resolution structural information has been obtained for the bacterial rhodopsin and bovine rhodopsin from two-dimensional cryo-electromicroscopic experiments (41, 42).

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The abbreviations used are: DPDPE, cyclic [α-penicillamine², n-penicillamine⁶]-enkephalin; SNC-121, (+)-4-[(α-R)-α-(25,5F)-4-propyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl|-N,N-diethylbenzamide; hDOR, human δ-opioid receptor.
For these reasons, we have designed a mutagenesis strategy based on the restoration of a lost function to identify amino acid sequence combinations critical to confer δ-selective ligand binding to an opioid receptor.

**EXPERIMENTAL PROCEDURES**

*Construction of the Library of Mutants—pcDNA3-hDOR consists of a 1.2-kilobase cDNA EcoRI-XhoI fragment of the δ-opioid receptor (23) sub-cloned at the EcoRI site of pcDNA3 (Invitrogen). Using unique site elimination (USE) mutagenesis (43), pcDNA3-hDOR was mutated to pcDNA3-δ291–300 by replacing 10 amino acids (positions 291–300) of the third extracellular loop of the δ-receptor with the corresponding amino acids from the μ-receptor. The selection primer was designed to mutate the unique PvuI site of pcDNA3-hDOR to a EcoRV site (5′-GCT CCT TCG GTC CTC GAT ATC TTG TCA GAA GTA AGT TGG C-3′) and primer δ291–300 (5′-GTC AGG CTG CTG AGC TTC CGG TCT GG TGC ATC GGC GCG CTC GGT TAC-3′) was used to produce the pcDNA3-δ291–300 chimera. The library of mutants of the δ291–300 chimera was produced by USE mutagenesis (43) using pcDNA3-δ291–300 as the parental vector. A degenerated primer, δ291–300 degenerated (5′-GTC TGG AGC CGG ATG AAC GAC GTC GTC ATT GGG GAG AAC GAC GCA TAC GGC TAT CAG CTG GAT ATC TTG TCA GAA GTA AGT TGG C-3′), sequences of the primers were 5′-CTG GGT TAC CTG GGT TAC GGC GCG CTC GGT TAC-3′, was designed to randomly and independently revert amino acids of the μ sequence to the original δ sequence (16,384 possible combinations). Each degenerated position contained an equal ratio of the nucleotide from the μ or the δ sequence. This degenerated primer was used with a selection primer EcoRV to PvuI (5′-GCT CCT TCG GTC CTC GAT ATC TTG TCA GAA GTA AGT TGG C-3′) to perform a mutagenesis reaction on pcDNA3-δ291–300. This synthesis mixture was transformed into Escherichia coli DH5α cells, and pools of clones were randomly selected. Plasmid DNA from each pool was isolated using QiAprep 8 plasmid kit (Qiagen, Chatsworth, CA) and used for transfection into HEK 293s cells.

*Site-directed Mutagenesis—Single and double point mutations were introduced into δ291–300 chimera using Clontech site-directed mutagenesis kit which is based on the method of Deng and Nickoloff (43). In a first mutant, we introduced a single mutation that converts tryptophan residue 300 to leucine residue (5′-CTG GTG GAC ATC GAC CGA GAA ACT ACG TTC CAG-3′). Another mutant of the chimera was constructed into which proline residue 291 was converted to arginine residue (5′-CTG GTG GAC ATC GAC CGA GAA ACT ACG TTC CAG ACT GTT TCT TGG CAC CTG TGC ATC GCG-3′). This synthesis mixture was used with a selection primer to obtain P291R, W300L, and P291R/W300L mutants, respectively.

*Cell Culture and Transfection Procedure—Human embryonic kidney 293s (obtained from Michael Matthew, Cold Spring Harbor) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transiently transfected according to the procedure of Chen and Okayama (44). Transfections were performed using 15 μg of expression vectors and 1 × 10^5 cells per 25-cm² flasks. Binding of δ-selective ligands (SNC-121 and DPDPE) to the transfected cells was monitored 48 h after transfection.

*Screening for Revertant Mutants That Bind δ-Selective Ligand—Pools containing 50 clones each were screened for the presence of revertant mutants with affinity for δ-selective ligands using a radioligand binding assay. Cells transfected with pools of the library were assayed 48 h post-transfection for the binding of δ-selective ligands ([3H]DPDPE (peptide agonist) and [3H]SNC-121 (non-peptide agonist) (45)). Glycerol stocks of E. coli transformants corresponding to positive pools were partitioned into smaller pools of 10 clones using a row/column strategy.

One hundred colonies from each positive pool were inoculated on a Petri dish using 10 rows × 10 columns pattern. After overnight incubation, the 10 colonies from each row and each column were pooled into 5 ml of Luria-Bertani (LB) broth and incubated overnight at 37 °C. DNA from each pool was prepared as described and transfected into 293s cells for ligand binding analysis. Colonies at the intersection of a positive column and a positive row were selected for sequencing and further pharmacological characterization.

The sequence of the revertants was determined by dideoxynucleotide chain termination method using T7-DNA polymerase (Pharmacia Biotech Inc.) and α-35S-DATP (DuPont NEN).

**FIG. 1. Experimental strategy for the restoration of function analysis.** δ291–300 chimera that has lost the ability to bind δ-selective ligand was used as a template in a random mutagenesis reaction. We used a degenerated primer to randomly revert back to the δ sequence some of the μ residues located between positions 291 and 300. This mutagenesis generated a library of more than 16,000 mutants that were separated in pools of 50 clones. The plasmid DNA from each pool of clones was isolated and transfected into HEK 293s cells. The presence of a receptor mutant within a pool was detected if the transfected cells expressed a binding site for the selective ligand. The pool was then gradually split to isolate the clone responsible for the ligand binding activity. The DNA sequence of many revertant clones was determined and compared to identify structural features common to such revertant clones.

*Radioligand Binding Assay—*For the receptor binding study, HEK 293s cells expressing pools of mutant receptors were harvested 48 h after transfection and resuspended in 1.5 ml of membrane buffer (50 mM Tris-HCl, pH 7.4, 320 mM sucrose). Cells were then frozen/thawed, and an aliquot was used for the radioligand binding assay. Cells (50 μl) were incubated in a final volume of 150 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 0.1% bovine serum albumin) with [3H]bremazocine (6.9 nM, specific activity 30 Ci/mmol) (DuPont NEN) (46), [3H]-labeled [3H]-bremazocine ([3H]bremazocine, [3H]DPDPE, or [3H]SNC-121. Nonspecific binding was determined using 10 μM naloxone. For competition experiments, duplicate contained ~2.5 μM [3H]bremazocine and 1 μM to 10 μM DPDPE or SNC-80. Reactions were terminated by filtration on polyethyleneimine-preculted GF/B Whatman filters. The filters were washed three times with ice-cold buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂) and dried. Bound radioactivity was counted using a scintillation counter. Specific binding was determined.
as the difference between binding in the absence or presence of an excess of unlabeled naloxone (10 μM). Curve fitting and analysis of the binding data were performed using the GraphPad Prism program version 1.03 (1994).

Three-dimensional Modeling—The three-dimensional model of human δ-opioid receptor was constructed following a general procedure to build G protein-coupled receptors. There are three steps in this procedure. First, we identified the transmembrane helical domains from sequence alignments of the opioid receptor subfamily. Using the identified sequences, we built the initial helices bundle and then searched for the maximum interactions among these seven helices using a mixed molecular dynamics and conformational search procedure with the restraints from the projection density maps of rhodopsin (41). Finally we added the extracellular loops obtained from the Protein Data Base based on the sequence homology analysis. The sequence of human δ-opioid receptor (47) (Genbank P41143) was first submitted to the TMAP procedure of EMBL (48) to search for the opioid receptor family and to identify the transmembrane helical regions. The assumption that the arginine and lysine residues are most likely at the end of helices (49) was further used to adjust the helical regions. The initial helix building, the sequence homology, and the final structure refinement were performed using Quanta/CHARMM (Biosym/MSI). The mixed molecular dynamics and conformational search procedure was developed in-house.

RESULTS

Experimental Strategy—Fig. 1 illustrates the mutagenesis strategy we have designed to identify residues critical for the binding of δ-selective ligands. This strategy relied on the restoration of a function rather than the loss of a function. A chimeric receptor unable to bind the δ-selective ligands was used as template in a mutagenesis reaction. The amino acid sequence over the 10-amino acid region. The primer was designed to allow each residue of this stretch to be either of the μ or the δ sequence. Owing to the design of the primer, some positions could also code for non-μ and non-δ residues (see Fig. 2B). Using the pcDNA3-δ/μ291-300 plasmid as a template and δ/μ291-300.degenerated as the mutagenic primer, we performed a mutagenic synthesis theoretically generating all the possible combinations of μ, δ, or non-μ, non-δ residues over the 10 amino acids of the third extracellular loop of δ/μ291–300 (Fig. 2B). This represents 16,384 possible combinations.

To evaluate the frequency of amino acid substitution over the targeted 10-amino acid stretch, 50 clones of the mutant receptor library were randomly selected and subjected to DNA sequencing. Sequence analysis of the clones showed that 90% of these clones were mutated and contained 48.3% amino acid substitution at a position where a combination of two residues was possible and a percentage of substitution increased to 76% at a position where a combination of four different residues was possible (Fig. 3). These results indicate that amino acid substitution occurred randomly and without any preference for one or the other sequence.

Construction of the δ/μ291-300 Chimera and Random Mutagenesis—Evidence from different groups has suggested that the third extracellular loop of δ-opioid receptors is involved in the binding of selective ligands (35–40, 50). A chimeric δ-opioid receptor in which 10 amino acids of the third extracellular loop (amino acids 291 to 300) were replaced by the corresponding amino acids from the μ receptor was constructed. HEK 293s cells were transfected with the plasmid DNA coding for the chimera, and radioligand binding assays using the nonselective [3H]bremazocine or δ-selective ligands [3H]SNC-121 and [3H]DPDPE were performed 48 h post-transfection. The radioligand binding properties of δ/μ291–300 chimera are shown in Fig. 2A and summarized in Table I. This chimera binds the nonselective opioid ligand bremazocine with the same affinity as the wild-type receptor (Fig. 2A and Table I). However, it does not bind δ-selective ligands such as [3H]DPDPE or [3H]SNC-121 (Fig. 2A and Table I).

We then randomly and independently substituted μ residues of this chimera with the corresponding δ residues. To this end, we used a degenerated primer (δ/μ291–300.deg) to mutate this 10-amino acid region. The primer was designed to allow each residue of this stretch to be either of the μ or the δ sequence. Owing to the design of the primer, some positions could also code for non-μ and non-δ residues (see Fig. 2B). Using the pcDNA3-δ/μ291–300 plasmid as a template and δ/μ291-300.degenerated as the mutagenic primer, we performed a mutagenic synthesis theoretically generating all the possible combinations of μ, δ, or non-μ, non-δ residues over the 10 amino acids of the third extracellular loop of δ/μ291–300 (Fig. 2B). This represents 16,384 possible combinations.

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**Table I**

Ligand binding characteristics of wild-type, chimeric, and mutated receptors

| Ligand     | B<sub>max</sub> (pmol/mg) | K<sub>d</sub> (nM) | K<sub>i</sub> (nM) |
|------------|------------------|----------------|----------------|
| [3H]bremazocine | 1.1 ± 0.19    | 3.4 ± 0.3     | 3.1 ± 0.3     |
| δ<sub>μ</sub>291–300 | 0.81 ± 0.05   | 2.2 ± 0.2     | 2.0 ± 0.0     |
| 2.40       | 0.43 ± 0.04    | 1.5 ± 0.2     | 6.2 ± 2.2     |
| 29.4       | 0.19 ± 0.002   | 2.2 ± 0.2     | 1.9 ± 0.7     |
| 37.5       | 0.29 ± 0.10    | 3.6 ± 0.4     | 2.4 ± 0.3     |
| 32.45      | 0.50 ± 0.15    | 1.3 ± 0.1     | 2.2 ± 0.5     |
| 32.55      | 0.30 ± 0.04    | 5.6 ± 0.6     | 1.6 ± 0.1     |
| 16.9.9     | 0.17 ± 0.04    | 3.8 ± 0.8     | 2.1 ± 0.4     |
| 16.9.18    | 0.67 ± 0.36    | 3.9 ± 0.4     | 1.9 ± 0.3     |
| 18.6.3     | 0.78 ± 0.17    | 3.1 ± 0.3     | 2.4 ± 0.2     |

*NB*, no detectable binding.

| δ<sub>μ</sub>291–300 mutations | R | D | P | L | V | T | V | S | W |
|-------------------------------|---|---|---|---|---|---|---|---|---|
| Mutation rate (%)             | 62 | 78 | 78 | 44 | 46 | 76 | 72 | 36 | 52 | 50 |

**Fig. 3.** Frequency of amino acid substitution over the targeted region. Fifty clones of the mutant receptor library were randomly selected and subjected to DNA sequencing. Sequence analysis of the clones showed that 90% contained mutations. On average five positions were mutated in each clone. The mutation rate at individual positions was approximately 50% at positions where two possible amino acids could be obtained (positions 291, 294, 295, 298, 299, and 300) and increased to 75% when four different amino acids were possible (positions 292, 293, 296, and 297).

**Screening of the Library—**Preliminary experiments were performed to determine the size of the pools where a single revertant would be detected using radioligand binding assays. We transfected HEK 293s cells with different dilutions of the wild-type hDOR expression vector corresponding to pools of 1–10,000 clones that would contain a single colony encoding a wild-type hDOR receptor. In this experiment, we observed that statistically significant specific binding can be detected using pools of 500 clones (one of which being hDOR) using [3H]SNC-121 (6 nM) or [3H]DPDPE (6 nM) as radioligands (data not shown). After performing a series of preliminary experiments to determine the ideal pool size, we decided to screen pools of 50 clones.

DNA from pools of 50 clones each were then transfected into HEK 293s cells according to the procedure of Chen and Okayama (44). Radioligand binding assays using δ-selective ligands were performed 48 h after transfection. Forty pools of 50 clones were transfected into HEK 293s cells and screened independently with [3H]DPDPE or [3H]SNC-121 in the presence or absence of 10 μM naloxone. From this first screen, 6 positive pools (pool 2, 16, 18, 29, 32, and 37) were selected since they displayed significant affinity for [3H]DPDPE and [3H]SNC-121. These δ-selective binding pools were subdivided into smaller pools using the row/column strategy described under “Experimental Procedures.” Each pool contained one revertant except pools 16 and 32 from which two positive clones were obtained, yielding a total of eight revertants (Table I).

**Ligand Binding Properties of the Revertant Mutants—**The plasmid DNAs encoding the eight revertant receptors were transfected into HEK 293s cells, and the transfected cells were analyzed 48 h post-transfection. Saturation binding experiments using [3H]SNC-121, [3H]DPDPE, and [3H]bremazocine were performed to determine the respective K<sub>d</sub> values for each of the revertants. Saturation binding experiments with [3H]bremazocine were used to evaluate binding site levels. The examination of B<sub>max</sub> values (Table I) indicates that mutations under study do not drastically modify receptor expression in HEK 293s cells. Expression levels of most of the mutants are not significantly modified. B<sub>max</sub> values of 29.4, 37.5, 32.55, 16.9.9 and hDOR(R291P) mutants are slightly decreased, but radioligand binding remains nevertheless easily detectable. All the identified revertants display affinity for the δ-selective ligand ([3H]SNC-121 and [3H]DPDPE) and nonselective ligand ([3H]bremazocine) similar to the wild-type δ-opioid receptor. Interestingly, all the revertants regained the ability to bind both [3H]SNC-121 and [3H]DPDPE. No statistical differences were noted between the K<sub>i</sub> values measured for the different revertants.

**Sequence Analysis of the Revertant Mutants—**The sequences of the revertant mutants were determined and are shown in Fig. 4. Analysis of the sequence of these revertants revealed that an arginine at position 291 and a leucine at position 300 (from the δ sequence) were present in all the revertants (Fig. 4). Amino acids from either the δ, μ, or non-μ/non-δ sequence were found at positions 291–294 suggesting that these residues are from non-δ amino acids that are reverted in all the binding mutants.
The mutations do not considerably alter the expression level. Competition assays using [3H]bremazocine as tracer and the δ-selective ligands DPDPE or SNC-80 as competitors were performed, and the Ki values are listed in Table II.

Double reversion to the δ sequence of residues located at positions 291 and 300 (d/m291–300 (P291R/W300L)) did not produce a further increase in affinity toward δ-selective ligands.

Similarly, reversion of proline 291 (m residue) to an arginine (d residue) (d/m291–300 (P291R)) did not increase affinity for δ-selective ligands DPDPE and SNC-80 as compared with the d/m291–300 chimera.

Three-dimensional Modeling of hDOR and Position of the Critical Residues—Three-dimensional computer modeling was used to gain some insight into the orientation of the residues that are present in all of the revertant mutants (Fig. 6). The three-dimensional model of the human δ-opioid receptor was constructed following a general procedure for G protein-coupled receptors that has been described under “Experimental Procedures.” In this model, the seventh transmembrane domain starts at valine 296 which is 5 residues ahead of leucine 300, and these 2 residues are separated by a hydrophobic region. According to our model, the arginine localized at positions 291 and 300 of the revertant mutants is in a hydrophobic environment which is consistent with previous predictions based on the hydrophilicity analysis. The hydrophilicity analysis of the revertant mutants was performed using MacVector program (version 4.1.4) from Kodak International Biotechnologies Inc. Values above the axis denote hydrophilic regions that may be exposed on the outside of the molecule; values below the axis indicate the hydrophobic regions that tend to be buried inside the molecule or inside other hydrophobic environments such as membranes. We have used the Kyte and Doolittle scale with a window size of 7 residues and an amphiphilicity window size of 11. Underlined amino acids are from δ sequence. Lowercase amino acids are from non-δ/non-μ sequence.

Table II

| Ligand binding characteristics of wild-type δ receptor and single or double point mutants | [3H]bremazocine | DPDPE SNC-80 |
|---------------------------------|-----------------|---------------|
| Bmax  | Kd   | Km  | Bmax  | Kd   | Km  |
| pmol/mg | nM  | nM  | pmol/mg | nM  | nM  |
| hDOR  | 1.1 ± 0.19 | 3.4 ± 0.3 | 5.4 ± 1.8 | 7.0 ± 0.7 |
| δ/μ291–300 | 0.8 ± 0.05 | 2.1 ± 0.2 | >1000 | >1000 |
| δ/μ291–300 (P291R) | 1.85 ± 0.23 | 3.5 ± 0.7 | >1000 | >1000 |
| δ/μ291–300 (W300L) | 0.65 ± 0.06 | 2.5 ± 0.3 | 72 ± 4 | 102 ± 7 |
| δ/μ291–300 (P291R/W300L) | 0.83 ± 0.06 | 3.0 ± 0.5 | 68 ± 11 | 78 ± 2 |
| hDOR (R291P) | 0.43 ± 0.05 | 2.4 ± 0.1 | 7.2 ± 2.2 | 9.4 ± 0.2 |
tion 291 (Arg-291) (shown in yellow in Fig. 6) points toward the outside of the receptor suggesting that arginine 291 does not interact directly with ligand. The leucine localized at position 300 (Leu-300) (shown in yellow in Fig. 6) faces the inner side of the binding pocket and could directly interact with the δ-selective ligand SNC-121 which is represented in red in this figure. The hydrophobic region from amino acids 295–300, represented in green in Fig. 6, is localized at the top of the seventh transmembrane domain.

**DISCUSSION**

In this paper we describe the design and use of a “restoration of function” mutagenesis strategy to identify residues of the human δ-opioid receptor involved in the binding of subtype-selective ligands. Leucine 300 has been identified as a critical residue, and we proposed that residues at this particular position in other opioid receptor subtypes may play a role of exclusion of δ-selective ligands.

First, we generated a chimeric receptor (δ/μ291–300) in which 10 amino acids of the third extracellular loop of the human δ-opioid receptor were replaced by the corresponding amino acid sequence of the μ-opioid receptor. This protein binds nonselective opioid ligands but is devoid of affinity for δ-selective ligands. Our results are in agreement with results from previous studies using κ/δ or δ/μ chimeric receptors that have shown that δ-selective ligands interact mainly with the region containing the sixth transmembrane domain and the third extracellular loop of the δ-opioid receptor (35–40, 50). In this study, we have delimited this region to 10 amino acids that are located between arginine residue at position 291 and leucine residue at position 300.

Using this chimeric construct as the template, we generated a library containing theoretically 16,384 mutants in which combinations of amino acids of the third loop were reverted to the corresponding δ sequence. Next, we used radioactive δ-specific ligands to select from this receptor library mutants that had regained the ability to bind δ-selective ligands with high affinity. Using this novel strategy, we showed that a leucine at position 300, a hydrophobic region (amino acids 295–300), and an arginine at position 291 of the human δ-opioid receptor were present in all revertants suggesting a possible role of these residues in the binding of δ-selective ligands.

The binding characteristics of the δ/μ291–300 chimera demonstrates that replacing amino acids 291–300 of the δ-opioid receptor by the corresponding amino acids of the μ-opioid receptor abolishes δ-selective binding while preserving nonselective opioid ligand binding. This suggests that the overall structure of the chimera is preserved and that δ amino acids 291–300 contribute to δ selectivity either by making specific contacts with the δ ligand or by inducing conformational change in the receptor that would favor migration of the δ ligand to a binding pocket located more deeply in the receptor. Another hypothesis developed by Metzger and Ferguson (51) could be applied in this model. They suggest that opioid ligands would bind to their receptors into a pocket formed by the transmembrane helices and this pocket would be common to all opioid receptor subtypes. Selectivity would be conferred by the extracellular loops that would act as a gate to allow the passage of certain ligands while excluding others. In our model, μ residues located at positions 291–300 of the chimera could inhibit the passage of δ-selective ligand to the transmembrane binding pocket.

Comparison of amino acid sequences of the selected revertants allows a number of observations to be made. All revertants have substituted tryptophan 300 and proline 291 (μ sequence) from δ/μ291–300 chimera with a leucine and an arginine (δ sequence), respectively, suggesting that these positions might play a role in determining δ specificity. To more precisely define the contribution of leucine 300 and/or arginine 291 to the restoration of δ-selective ligand binding, these residues were mutated singly or in combination in δ/μ291–300 chimera.

δ/μ291–300 is devoid of any detectable affinity for δ ligands. The reversion of tryptophan 300 (μ residue) to a leucine (δ residue) in the construct (δ/μ291–300 (W300L)) partially restores the affinity for both δ-selective ligands DPDPE (Kᵢ = 72 nM) and SNC-80 (Kᵢ = 102 nM) (Table II). Nevertheless, these Kᵢ values remain 15 times higher than observed for the wild-type hDOR. The presence of a leucine at position 300 is not an
absolute requirement for δ-selective ligand binding, since a mutant of the δ-opioid receptor in which this leucine residue is substituted for an alanine binds SNC-80 with wild-type affinity.2 It appears that the absence of tryptophan at position 300 is more important than the presence of a leucine. It is conceivable that the presence of a bulky tryptophan residue at position 300 blocks the access of δ ligands to their docking site. Our three-dimensional model of the receptor (Fig. 6) suggests that the leucine at position 300 points toward the inside of the binding pocket. Therefore its replacement by a tryptophan would obstruct the access to the central pore of the receptor where the ligand docking site is likely located. These observations are in agreement with a recently proposed hypothesis (51) suggesting that the selectivity within the opioid receptor family may be imparted through a mechanism of exclusion, rather than specific pharmacore recognition within the extracellular loops.

Single reversion of proline 291 (μ residue) to arginine (δ residue) is not sufficient to restore the binding of δ-selective ligand (δμ291–300 (P291R)) (Table II). The tryptophan residue at position 300 is still present in this construction and may inhibit binding of δ-selective ligands. However when mutations reverting tryptophan 300 to a leucine and proline 291 to an arginine are introduced simultaneously in δμ291–300 chimera, there is no increase in binding affinity as compared with single reversion of tryptophan 300 to leucine (δμ291–300 (W300L)). This result indicates that in this sequence context, arginine 291 does not improve δ-selective binding. Therefore, the possible involvement of arginine 291 in the binding of δ-selective ligands remains unclear and has to be elucidated.

A mutant of hDOR with an alanine residue at position 291 instead of an arginine binds δ-selective ligand DPDPE and SNC-80 with wild-type affinity suggesting that arginine 291 (δ residue) is not critical for the binding of δ-selective ligand (35). However, the adjacent residue at position 292 is also an arginine which may compensate for the substitution at position 291. This interpretation is supported by the observation of Wang and co-workers (50) that has shown that a double mutation of arginine 291 and 292 abolishes the ability to bind DSLET (a δ-selective ligand) while retaining nonselective ligand binding properties.

We observed that residues 295–300 were hydrophobic in all the selected revertants. This result is supported by the work of Valiquette et al. (35) where they have shown that the valine 296 and the valine 297 residues of the hDOR are involved in the binding of δ-selective ligand. The present study suggests that it is the overall hydrophobic character of this region rather than its specific primary amino acid sequence that is important for δ-selective binding since the primary sequence of most revertants is divergent from the δ receptor sequence.

Amino acids 292–294 do not seem critical for the binding of δ-selective ligands. Indeed, binding of [3H]DPDPE and [3H]SNC-121 is observed with mutants bearing δ-, μ-, or non-δ or non-μ amino acids at positions 292, 293, and 294, suggesting that strict residue identity is not required at these positions for δ-selective binding.

The restoration of function strategy we have used presents some advantages over the traditional “loss of function” strategy. In the traditional mutagenesis strategy, the residues that are identified as critical are those causing a loss of binding function when mutated. In interpreting the results from such experiments, one needs to explain the reasons for the loss of function which could be due to the substitution of a residue essential for ligand binding, to a low level of expression of the mutant, a decrease in the stability of the mutant receptor, or inefficient traffic to the cell surface. By using careful controls, one can eliminate possible explanations, but there often remain some possibilities for misinterpretation. Unlike the traditional mutagenesis strategy, which analyzes the contribution of a single amino acid, the restoration of function strategy permits the identification of multiple combinations of residues thus allowing a specific function to be restored. Moreover, this positive approach allows us to identify nonessential positions or positions that can tolerate various substitutions. We thus have observed in this study that residues at positions 292–294 were not critical for the binding of δ-selective ligand since μ, δ, or non-μ/non-δ residues with different physicochemical properties were found at these positions. Finally, this positive approach can identify specific physicochemical properties (like hydrophobic characteristics) of a region or area required for the function. This situation is observed when residues are not reverted to a specific sequence but to residues sharing similar physicochemical characteristics.

In this study, we have developed a novel and efficient method for the analysis of the structure-function of the opioid receptors or possibly any G protein-coupled receptor. This method is based on a positive approach that allows identification of positions within the receptors that are essential, deleterious, or neutral for the interaction with different ligands.

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