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Research report

Purification and mass spectrometric analysis of the δ opioid receptor

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Abstract

A mouse δ opioid receptor was engineered to contain a FLAG epitope at the amino-terminus and a hexahistidine tag at the carboxyl terminus to facilitate purification. Selection of transfected human embryonic kidney (HEK) 293 cells yielded a cell line that expressed the receptor with a B_{max} of 10.5 pmol/mg protein. [^{3}H]Bremazocine exhibited high affinity binding to the epitope-tagged δ opioid receptor with a K_D of 1.4 nM. The agonists DADL, morphine, and DAMGO competitively inhibited bremazocine binding to the tagged δ receptor with K_I's of 0.9, 370, and 620 nM, respectively. Chronic treatment of cells expressing the epitope-tagged δ receptor with DADL resulted in downregulation of the receptor, indicating that the tagged receptor retained the capacity to mediate signal transduction. The δ receptor was solubilized from HEK 293 cell membranes with *n*-dodecyl-β-D-maltoside in an active form that maintained high affinity bremazocine binding. Sequential use of Sephacryl S300 gel filtration chromatography, wheat germ agglutinin (WGA)-agarose chromatography, immobilized metal affinity chromatography, immunoaffinity chromatography, and SDS/PAGE permitted purification of the receptor. The purified δ opioid receptor was a glycoprotein that migrated on SDS/PAGE with an apparent molecular mass of 65 kDa. MALDI-TOF mass spectrometry was used to identify and characterize peptides derived from the δ opioid receptor following in-gel digestion with trypsin, and precursor-derived ms/ms confirmed the identity of peptides derived from enzymatic digestion of the δ opioid receptor.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Opioid receptors

Keywords: Opioid; Purification; Mass spectrometric analysis; Morphine; Enkephalin; Mass spectrometry; Affinity chromatography

1. Introduction

Opioid receptors mediate the effects of endogenous opioid peptides in the central, peripheral, and enteric nervous systems and are also the molecular targets of opioid drugs, such as heroin, morphine, fentanyl, and methadone [5]. The three types of opioid receptors, μ, δ, and κ, are members of the G protein-coupled receptor superfamily [10,28]. Like other G protein-coupled receptors, opioid receptors contain seven transmembrane domains, have extracellular amino termini that contain sites for N-linked glycosylation (Asn-X-Ser/Thr), and have carboxyl termini located intracellularly. Opioid ligands approach and bind the receptor from the extracellular side, and agonist-induced opioid receptor activation results in the coupling to G proteins on the intracellular aspect of the plasma membrane. Opioid-induced signal transduction is mediated through a variety of effectors, including adenylyl cyclase, K^+ channels, Ca^{2+} channels, MAP kinase, and phosphatidylinositol 3-kinase [3,4,16,24,25].

Abbreviations: DADL, [D-Ala^2, D-Leu^5]enkephalin; DAMGO, [D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]enkephalin; HEK, human embryonic kidney; MALDI, matrix-assisted laser desorption ionization; ms/ms, tandem mass spectrometry; Ni-NTA, nickel–nitrilotriacetic acid; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TOF, time of flight; WGA, wheat germ agglutinin

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Chronic use of opioid drugs leads inevitably to physical dependence, which becomes evident when prolonged opioid drug use is terminated, resulting in a withdrawal syndrome lasting from several days to weeks [13]. Chronic opioid use also induces a state of tolerance, in which the pharmacological efficacies of agonists are attenuated. Intensive research efforts over the last several decades have indicated that opioid addiction is complex, and probably involves neural plasticity associated with alterations in the strength of neural circuits, changes in gene expression, and alterations in opioid-regulated signaling pathways [15,20].

Short-term agonist activation of opioid receptors leads to an adaptive response to acute effects that is referred to as receptor desensitization. It has been shown that μ and δ opioid receptors are phosphorylated by G protein-coupled receptor kinases following agonist treatment [21,31] and are internalized in a dynamin-dependent process via clathrin-coated pits [8]. Overexpression of arrestin or G protein-coupled receptor kinase increases agonist-induced opioid receptor internalization [29,31]. Morphine analgesia is enhanced in knockout mice lacking β-arrestin 2, pointing to a role for β-arrestin 2 in desensitization of the opioid receptor [1]. In mice lacking β-arrestin 2, chronic morphine treatment does not cause desensitization of the μ opioid receptor, and the knockout mice do not develop tolerance to the analgesic effects of morphine [2]. Endosome-associated receptors can be resensitized via dephosphorylation by protein phosphatases and recycled back to the plasma membrane, or be proteolytically degraded within the cell [11].

Chronic opioid administration leads to receptor down-regulation, as a result of proteolytic degradation of the receptors [17,23]. It is probable that agonist-induced down-regulation of opioid receptors contributes to opioid tolerance. The mechanism for G protein-coupled receptor proteolysis has been generally assumed to involve internalization and trafficking from endosomes to lysosomes; however, evidence that the ubiquitin/proteasome pathway plays a prominent role in agonist-induced opioid receptor downregulation has been reported [7]. In that study, proteasome inhibitors blocked agonist-induced downregulation of μ and δ opioid receptors, while inhibitors of calpain, caspases, and lysosomal cathepsins had no effect. It was also shown that μ and δ opioid receptors are ubiquitinated, which serves to target protein substrates to the proteasome complex. Downregulation of the human κ opioid receptor was also partially attenuated by proteasome inhibitors [18]. The ubiquitin/proteasome pathway also mediates degradation of improperly folded δ opioid receptors, as part of the quality control system for newly synthesized proteins in the endoplasmic reticulum [22]. Observations on the involvement of the ubiquitin/proteasome system in opioid receptor turnover have recently been extended to other G protein-coupled receptors, including the β2-adrenergic receptor [26], the CXCR4 chemokine receptor [9,19], and the CCR5 chemokine receptor [9].

In this study, we report the solubilization and purification of the δ opioid receptor expressed in HEK 293 cells, and the identification and characterization of peptides derived from proteolytic digestion of the purified receptor using MALDI-TOF and TOF-TOF ms/ms mass spectrometry.

2. Materials and methods

2.1. Construction of hexahistidine-tagged opioid receptors

A plasmid encoding the δ opioid receptor with a FLAG epitope at the N-terminus (kindly provided by Dr. Mark von Zastrow, UCSF) was used as a PCR template to generate a receptor that also contained a hexahistidine tag at its C-terminus. Oligonucleotide primers employed for the PCR were 5′-GCC ATG AAG ACG ATC ATC GCC, which was the sense primer used to amplify the 5′-end of the Flag-tagged δ opioid receptor and 5′-GGC GGC AGC GCC ACC GCC CGG, an antisense primer that deletes the termination codon from the 3′-end of the δ opioid receptor open reading frame. The PCR product was inserted into the eukaryotic expression vector, pcDNA3.1/V5-His-TOPO (Invitrogen). Deletion of the stop codon at the 3′-end of the δ receptor open reading frame allowed it to be placed in frame with a plasmid open reading frame encoding the V5-epitope and hexahistidine tag. The recombinant plasmid encoding the FLAG- and His-tagged δ opioid receptor was used to transform TOP10 *Escherichia coli*, and positive colonies were screened by PCR. Plasmids were purified on Qiagen columns and then submitted to the Molecular Resource Facility at UMDNJ-NJMS for DNA sequence verification of the entire receptor open reading frame.

2.2. Cell culture and transfection

HEK 293 cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. HEK 293 cells were transfected with the receptor expression plasmid using Lipofectamine (Invitrogen). Cells expressing the epitope-tagged δ opioid receptor were selected in media containing 1 mg/ml G418 (Life Technologies, Gaithersburg, MD).

2.3. Membrane preparation and radioligand binding assays

HEK 293 cells expressing the epitope-tagged δ opioid receptor were grown to near confluence in 150 mm diameter dishes. For membrane preparations, the culture medium was aspirated and cells were harvested in 50 mM Tris–HCl buffer, pH 7.5. The cell suspension was homogenized with a Tekmar tissuemizer (Cincinnati, OH), then centrifuged at 100,000 × *g* for 30 min. The membrane pellet was washed three times in Tris buffer and then

resuspended by homogenization in 0.32 M sucrose/50 mM Tris–HCl, pH 7.5, and the crude membrane preparation was stored at −80 °C.

Opioid receptor binding assays were conducted in duplicate with membrane preparations diluted in 50 mM Tris–HCl buffer, pH 7.5. [9-^{3}H](−)Bremazocine (specific activity 26.6 Ci/mmol, NEN, Boston, MA) was used as the radioligand. Following a 1-h incubation at 0 °C, assays were terminated by filtration through Whatman GF/B filters. Filters were submersed in Ecoscint liquid scintillation cocktail (National Diagnostics, Somerville, NJ) prior to determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. Residual bremazocine binding that was not displaced by 10 μM cyclazocine was defined as nonspecific binding and was subtracted from total binding to calculate specific binding. Receptor binding data were analyzed by nonlinear regression using Prism 3.0 c (GraphPad Software, San Diego, CA). Protein concentrations were determined with the BioRad *DC* protein assay (Hercules, CA), using bovine serum albumin as the standard.

2.4. Immunoblotting

For detection of the δ receptor by immunoblotting, samples were heated at 40 °C for 5 min in SDS/PAGE gel loading buffer (BioRad), resolved by SDS/PAGE using 12% gels, then transferred to Immobilon P^{SQ} PVDF membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h in 3% dried milk, 0.1 M Tris–HCl, pH 7.5, 1% BSA, 1 M glucose, 10% glycerol, 1 mM $CaCl_2$, 0.5% Tween 20, 0.005% thimerosal, followed by overnight incubation at 4 °C with mouse anti-FLAG M1 monoclonal antibody (Sigma). Membranes were then washed and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature and developed using CDP-Star Western blot chemiluminescence reagent (NEN Life Sci. Products, Boston, MA). Kodak BioMax MR film was used to capture chemiluminescence.

2.5. Solubilization

Washed membrane preparations derived from 50 cell culture plates containing 1–2 mg protein/ml were extracted for 30 min on ice with 1% *n*-dodecyl-β-D-maltoside (Anatrace, Maumee, OH), 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, and a protease inhibitor cocktail containing *N*-benzyloxycarbonyl-leucyl-leucyl-leucinal, 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64d, bestatin, leupeptin, and aprotinin (Sigma). Detergent extracts were centrifuged at 100,000 × g for 1 h in a Beckman L-70 ultracentrifuge and the supernatant was used for binding assays and receptor purification. Ligand binding to soluble receptors was determined using a polyethylene glycol precipitation assay as described previously [12]. Briefly, soluble receptors were incubated in duplicate 0.25 ml aliquots for 1 h at 0 °C with [^{3}H]bremazocine (0.05–10 nM) in the presence and absence of 10 μM cyclazocine, then precipitated by the addition of 0.5 ml 25% polyethylene glycol and 0.5 ml 0.1% γ-globulin in 50 mM Tris–HCl, pH 7.5, filtered through Whatman GF/B filters, and washed with 8% polyethylene glycol. Filter-bound radioactivity was determined by liquid scintillation counting. Saturation curves were analyzed by nonlinear regression using GraphPad Prism 3.0 c.

2.6. Gel filtration chromatography

All chromatographic procedures were performed in a cold room. Gel filtration chromatography was performed using Sephacryl S300 HR resin (Pharmacia) packed in a 2.6 × 90 cm column. The ultracentrifugal supernatant was prelabeled with 2 nM [^{3}H]bremazocine for 1 h at 0 °C prior to loading. The column was eluted at a flow rate of 100 ml/h with column buffer (0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5% glycerol). The column effluent was monitored for absorbance at 280 nm with an LKB Uvicord SII detector and the signal was sent to a Kipp and Zonen BD41 chart recorder. Fractions were collected at 4 min intervals, and 0.1 ml aliquots of each fraction were quantified by liquid scintillation counting to determine the elution position of the [^{3}H]bremazocine/receptor complex. The Sephacryl S300 column was calibrated with the following protein standards: carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, apoferritin, and thyroglobulin (Pharmacia).

2.7. WGA–agarose chromatography

Sephacryl S300 fractions containing the ligand/receptor complex were mixed batch-wise for 18 h at 4 °C with 4 ml of washed WGA–agarose (Vector Laboratories). The receptor/WGA suspension was transferred to a 2.5 × 25 cm chromatography column, the resin was washed with 80 ml of column buffer, and the δ receptor was eluted in 15 ml of column buffer containing 0.5 M *N*-acetylglucosamine.

2.8. Immobilized metal affinity chromatography

Fractions containing the δ receptor from lectin chromatography were pooled and mixed batch-wise for 18 h at 4 °C with 5 ml of washed nickel–nitrilotriacetic acid (Ni-NTA) matrix (Qiagen). The receptor/Ni-NTA suspension was applied to a 2.5 × 25 cm column, and the matrix was washed with 150 ml of column buffer. The column was washed sequentially with column buffer containing 10 mM imidazole, then 20 mM imidazole, and the epitope-tagged δ receptor was eluted with column buffer containing 250 mM imidazole.

2.9. Immunoaffinity chromatography

The δ receptor was purified further using immunoaffinity chromatography, employing the anti-FLAG M1 monoclonal

antibody bound to agarose beads (Sigma). Eluates from the Ni-NTA chromatography were pooled and incubated with 1 ml of the washed M1-agarose for 18 h at 4 °C with gentle mixing. The receptor/M1 bead suspension was transferred to a 1 × 10 cm column, the column was rinsed with 30 volumes of column buffer, and then eluted with 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 20 mM EDTA, and 5% glycerol.

2.10. SDS/PAGE

Fractions containing the purified δ receptor from the M1 agarose column were pooled and concentrated from 3 ml to 40 μl using Amicon Centricon 30 filters. The concentrated sample was mixed with 5× SDS/PAGE gel loading dye (225 mM Tris–HCl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 250 mM DTT) and heated at 40 °C for 5 min. The purified receptor was resolved on a 12% SDS–PAGE gel, then stained with Coomassie blue. The 65-kDa δ receptor band was the predominant band in the gel and was excised with a sterile stainless steel scalpel and transferred to a 1.5-ml microcentrifuge tube.

2.11. In-gel digestion with trypsin

The gel fragment was diced into 1 mm^3 fragments and washed 3 times with 400 μl 50% acetonitrile/25 mM ammonium bicarbonate, pH 8.0, for 15 min to remove the Coomassie blue dye. Gel fragments were then soaked in 100% acetonitrile for 5 min, acetonitrile was removed, and the sample was dried in a SpeedVac (Savant) for 20 min at room temperature.

Prior to enzymatic digestion, gel fragments were reduced with 10 mM dithiothreitol in 50 mM ammonium bicarbonate at 60 °C for 45 min, then alkylated with 55 mM iodoacetamide in ammonium bicarbonate for 30 min, at room temperature, in darkness and dehydrated as described above. The reduced and alkylated gel slices were rehydrated in 50 mM ammonium bicarbonate, dehydrated once more with acetonitrile, and then hydrated with 20 μl of 50 mM ammonium bicarbonate, pH 8.3, containing 200 ng of trypsin (Promega, sequencing grade). Once this solution was fully absorbed by the gel slices, enzyme-free ammonium bicarbonate buffer was added until the gel pieces were covered. The samples were digested for 18 h at 37 °C, extracted with 50% acetonitrile/5% trifluoroacetic acid for 30 min, sonicated for 5 min, and then reduced in volume to 5 μl in a SpeedVac.

2.12. Mass spectrometry

Tryptic peptides were desalted using C_{18} ZipTips (Millipore), eluted with 1 μl 50% acetonitrile/0.1% trifluoroacetic acid, and loaded onto a sample plate prespotted with 0.5 μl sample loading matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) for analysis. Mass spectra were collected on an Applied Biosystems Voyager MALDI DE-PRO and an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics.

3. Results

3.1. Expression of the epitope-tagged δ opioid receptor and analysis of ligand affinities

Delta receptor constructs containing the FLAG epitope at the amino terminus and a hexahistidine tag at the carboxyl terminus expressed at high levels in HEK 293 cells. One clonal cell line was selected that displayed a B_{max} of 10.5 ± 0.6 pmol/mg protein. [^{3}H]Bremazocine exhibited high affinity binding to the epitope-tagged receptor with an apparent dissociation constant (K_D) of 1.4 ± 0.2 nM (Fig. 1A).

In order to provide further evidence that the ligand binding site of the epitope-tagged receptor was similar to the wild type, the affinities of several other opioid ligands were determined by competition analysis. The agonists DADL, morphine, and DAMGO competitively inhibited bremazocine binding to the engineered δ receptor with K_I's of 0.9, 370, and 620 nM, respectively (Fig. 1B). These values are in excellent agreement with the affinities of these compounds for the wild-type δ receptor published previously by this laboratory [6].

3.2. Agonist-induced downregulation of the epitope-tagged δ opioid receptor

Confirmation that the epitope-tagged δ receptor retained the capacity to be activated and transduce signals was obtained by studying agonist-induced downregulation of the receptor. Analysis of saturation curves indicated that incubation of the HEK 293 cell line expressing the epitope-tagged δ receptor for 18 h with 1 μM DADL induced a >90% decrease in B_{max} relative to untreated controls (Fig. 1C), with no significant change in the affinity of bremazocine for the receptor. Similar data were obtained when receptor levels were assayed by Western blot analysis (Fig. 1C, inset). The magnitude of the DADL-induced downregulation was very similar to that previously reported by this laboratory [7], indicating that the presence of the hexahistidine tag at the C-terminus of the δ receptor did not interfere with agonist-induced receptor activation and signal transduction.

3.3. Receptor purification

The nonionic detergent, *n*-dodecyl-β-D-maltoside, was employed for solubilization of the δ receptor, based on its ability to solubilize the receptor in an active form and its compatibility with subsequent mass spectrometric analysis

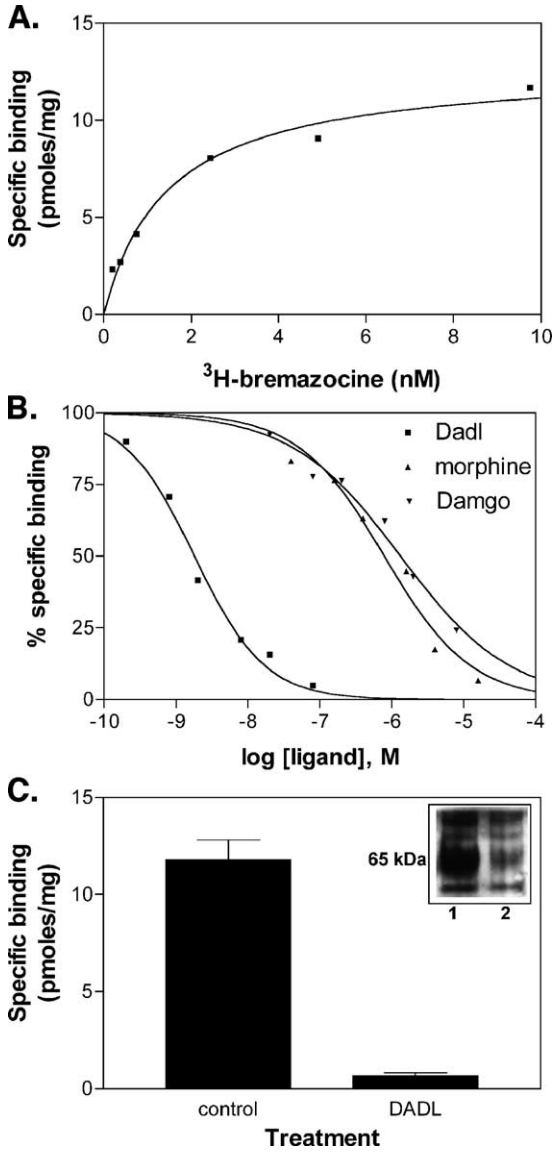

Fig. 1. Characterization of the epitope-tagged δ opioid receptor. (A) Saturation curve of [^{3}H]bremazocine binding. Membrane preparations derived from HEK 293 cells stably expressing the FLAG- and His-tagged δ opioid receptor were incubated with various concentrations of [^{3}H]bremazocine in the presence and absence of 10 μM cyclazocine for 60 min at 0 °C. Specific binding was determined following rapid filtration through Whatman GF/B filters. Results from five experiments indicated a K_D = 1.4 ± 0.2 nM, and a B_{max} = 10.4 ± 0.6 pmol/mg protein. (B) Competition analysis of ligand binding to the epitope-tagged δ opioid receptor. Membrane aliquots were incubated with 1.2 nM [^{3}H]bremazocine in the presence and absence of varying concentrations of DADL, morphine and DAMGO for 60 min at 0 °C. Results from five experiments yielded K_I's of 0.9, 370, and 620 nM, for DADL, morphine, and DAMGO, respectively. (C) Agonist-induced down-regulation of the epitope-tagged δ receptor. HEK 293 cells expressing the epitope-tagged δ receptor were incubated at 37 °C in serum-free medium for 18 h in the presence and absence of 1 μM DADL. Washed membrane preparations were used for saturation analysis using [^{3}H]bremazocine. B_{max} values for control and DADL-treated samples were 11.6 ± 1.5 and 0.65 ± 0.2 pmol/mg protein, respectively (n = 5). (Inset) Cells were treated for 18 h in the presence and absence of 1 μM DADL, extracted with 1% n-dodecyl-β-D-maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, and assayed by Western blot analysis using the anti-FLAG M1 antibody for detection. Lane 1: control extract (20 μg protein); lane 2: DADL-treated extract (20 μg protein).

of the purified protein. The affinity of bremazocine for the dodecyl maltoside-solubilized δ receptor was 3.8-fold lower than the affinity for the membrane-bound receptor (Table 1). The B_{max} of the soluble preparation was 8.7-fold lower than the B_{max} of the membranes, indicating that the solubilization yield of active receptors was approximately 12%. When the solubilization yield of total immunoreactive receptor was estimated by Western blot analysis, however, it appeared that the actual receptor yield was much greater, approaching 100%.

The ligand binding activity of the detergent-solubilized δ receptor was labile when stored at 4 °C. The loss of activity exhibited biphasic kinetics: approximately 60% of the δ receptors inactivate with a half-life of 10 h, while the remaining binding activity was lost with a half-life of 160 h (data not shown). This time-dependent loss of receptor binding activity rendered quantification of receptor yield and purity by ligand binding unsuitable; therefore, receptor purification was followed routinely by Western blotting using the anti-FLAG M1 monoclonal antibody.

The ultracentrifugal supernatant containing the dodecyl maltoside-solubilized δ receptor was fractionated using gel filtration chromatography, which also provided an estimate of the size of the solubilized ligand/receptor complex. Initially Sephacryl S200 resin was used; however, it was found that the detergent-solubilized δ receptor eluted coincident with the void volume, which contained the majority of protein (data not shown). Based on this observation, Sephacryl S300 resin was employed. With this matrix, the solubilized δ receptor complex was well separated from the protein in the void volume (Fig. 2). Recovery of the prebound receptor was typically 75–85%, with a purification factor of threefold. When the receptor complex was prelabeled with ^{3}H-bremazocine in the presence of a 1000-fold molar excess of unlabeled cyclazocine, the small peak of radioactivity that eluted in the void volume (fractions 26–30) and the major radioactive peak representing the bremazocine/receptor complex in fractions 32–41 were completely displaced by the excess unlabeled

Table 1
Characterization and yield of detergent-solubilized δ opioid receptors

	K_D (nM)	B_{max} (pmol/mg)	% B_{max}
Membrane fraction	1.4 ± 0.2	10.4 ± 0.6	
Soluble fraction	5.3 ± 0.6	1.2 ± 0.3	12

Membranes from HEK 293 cells expressing the epitope-tagged δ opioid receptor were solubilized in 1% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, then centrifuged at 100,00 × g for 1 h. Binding assays were conducted with the membrane fraction and the soluble supernatant following a 1-h incubation at 0 °C with [3H]bremazocine (0.1–10 nM) in the presence and absence of 10 μM cyclazocine. Soluble receptors were precipitated as described previously [12] by the addition of 11.4% polyethylene glycol and 0.045% γ-globulin in 50 mM Tris–HCl, pH 7.5, filtered through Whatman GF/B filters, washed with 5 ml 8% polyethylene glycol, and filter-bound radioactivity was determined by liquid scintillation counting. Saturation curves were analyzed by nonlinear regression using GraphPad Prism 3.0 c. Data are derived from three experiments.

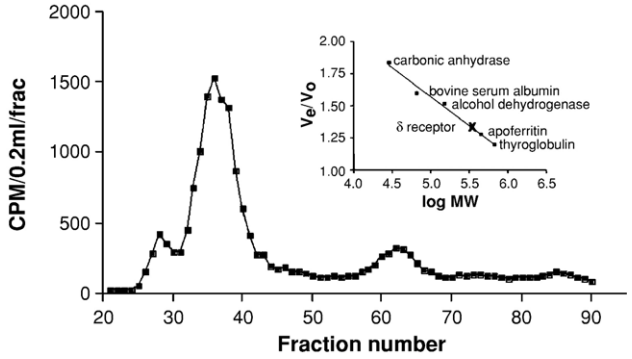

Fig. 2. Gel filtration chromatography of the δ opioid receptor. The solubilized δ opioid receptor was incubated with 2 nM [^{3}H]bremazocine for 1 h at 0 °C, and the sample was applied to a 2.6 × 90 cm Sephacryl S300 column and eluted with 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% glycerol at a flow rate of 100 ml/h. Fractions were quantified by liquid scintillation counting to determine the elution position of the [^{3}H]bremazocine/receptor complex. The major peak of 280 nm absorbance eluted in the void volume, fractions 26–30 (not shown), and the major peak of the ligand/receptor complex eluted in fractions 32–41. (Inset) Molecular weight estimation of the ligand-occupied δ opioid receptor. The Sephacryl S300 column was calibrated with carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, apoferritin, and thyroglobulin (Pharmacia). The calculated molecular weight of the ligand/δ receptor complex was 338,000.

ligand (data not shown). These data provided strong evidence that the major peak of radioactivity in fractions 32–41 represented the ligand-bound receptor complex. This conclusion was confirmed by Western blotting (see Fig. 3, below).

Calibration of the Sephacryl S300 column with protein standards indicated that the detergent-solubilized δ receptor complex migrated with an apparent molecular mass of approximately 340 kDa (Fig. 2, inset). The theoretical molecular weight of the epitope-tagged δ receptor without posttranslational modifications is 46,236. The solubilized receptor is contained within *n*-dodecyl-β-D-maltoside micelles, which have an average molecular mass of 40–47 kDa (CMC = 0.12 mM, aggregation number = 78–92). It was obvious that under gel filtration conditions, protein aggregation was occurring involving either multimers of the receptor itself or association with other proteins.

The next step in the purification procedure was WGA–agarose chromatography, and aliquots of the column input, flow through fraction, and *N*-acetylglucosamine eluate were resolved on duplicate SDS/PAGE gels. One gel was analyzed by Western blotting using the anti-FLAG M1 antibody for detection (Fig. 3A), and the other was stained with Coomassie blue (Fig. 3B). The predominant δ receptor immunoreactivity following SDS/PAGE appeared as a broad diffuse band with a range of apparent molecular mass from 50 to 70 kDa, presumably due to posttranslational modifications of the receptor, as suggested previously [7]. In addition, two other bands with apparent molecular mass of approximately 44 and 37 kDa were present, which did not bind to the WGA matrix as avidly as the upper band. The characterization of the molecular differences between the three immunoreactive species must await further analyses. The 50–70 kDa epitope-tagged δ opioid receptor bound to the WGA resin efficiently, based on the paucity of receptor immunoreactivity in the flow through fraction (lane 2), and was eluted effectively with *N*-acetylglucosamine (lane 3). The amount of protein loaded in lane 1 (40 μg) represented 0.04% of the total solubilized receptor preparation and the amount of protein loaded in lane 3 (4 μg) represented 0.5% of the eluted fraction. Quantification of the respective immunoreactive bands in Fig. 3A indicated that the total immunoreactivity in lane 3 was fourfold greater than the immunoreactivity in lane 1. Accordingly, we estimated that the yield of receptor from the WGA column was approximately 30–35%. Given that the WGA eluate in lane 3 contained four times the amount of immunoreactive δ opioid receptor relative to the input with 1/10th the amount of protein, we estimated that the purification factor for the WGA chromatography was approximately 40-fold.

The next step in the purification scheme utilized immobilized metal affinity chromatography with a Ni-NTA matrix. Aliquots of the various fractions were assayed following SDS/PAGE by immunoblot analysis using the anti-FLAG M1 antibody for detection (Fig. 4). Comparison of the immunoreactive receptor signals in lanes 1 and 2 (corresponding to the column input and flow-through fractions, respectively) suggested that >80%

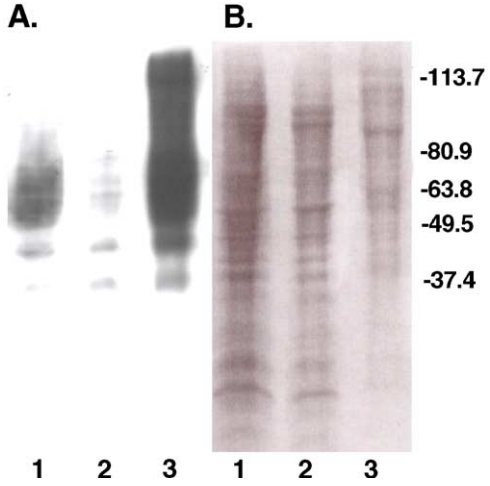

Fig. 3. Purification of the δ opioid receptor using WGA lectin chromatography. Fractions containing the ligand/receptor complex from Sephacryl S300 chromatography were pooled and incubated batch-wise for 18 h at 4 °C with wheat germ agglutinin-agarose beads. The suspension was transferred to a chromatography column, the flow through fraction was collected, the resin was washed with 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5% glycerol, and the δ receptor was eluted in wash buffer containing 0.5 M *N*-acetylglucosamine. (A) Immunoblot using anti-FLAG M1 antibody for detection. (B) Coomassie blue stain. Lane 1: input to the WGA resin (40 μg protein), lane 2: flow-through fraction (35 μg protein), lane 3: fraction eluted with *N*-acetylglucosamine (4 μg protein). Protein molecular weight markers are indicated to the right of the gels.

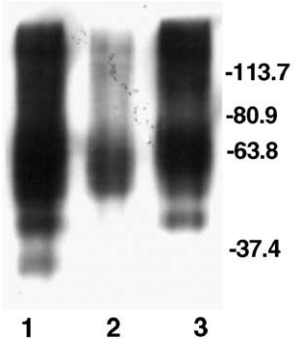

Fig. 4. Purification of the δ opioid receptor using immobilized metal affinity chromatography. The WGA eluate was incubated batch-wise for 18 h at 4 °C with the Ni-NTA matrix. The receptor/Ni-NTA suspension was transferred to a chromatography column, the flow through fraction was collected, the resin was washed with 150 ml of 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% glycerol. The Ni-NTA matrix was then washed sequentially with wash buffer containing 10 mM imidazole, then 20 mM imidazole, prior to elution of the epitope-tagged δ receptor with wash buffer containing 250 mM imidazole. Aliquots of the various fractions were assayed by Western blot analysis using the anti-FLAG M1 monoclonal antibody for detection. Lane 1: 0.5% of the input to the Ni-NTA column (4 μg protein); lane 2: 0.5% of the Ni-NTA flow through fraction (3.5 μg protein); lane 3: 0.5% of the 250 mM imidazole eluate (0.12 μg protein).

of the δ receptor bound to the Ni-NTA matrix, and the signal in lane 3 suggested that the majority of the bound receptor was eluted from the column with 250 mM imidazole. The strength of the signal in lane 3 was approximately equal to that in lane 1, with only 3% of the amount of protein, suggesting that a purification factor of at least 30-fold was achieved using immobilized metal affinity chromatography.

Following Ni-NTA chromatography, the δ receptor was purified further using immunoaffinity chromatography, utilizing the anti-FLAG M1 monoclonal antibody bound to agarose beads. Binding to the M1 antibody is Ca^{2+}-dependent, therefore the bound receptor was eluted with buffers containing 10 mM EDTA to chelate the $CaCl_2$ that is present in the binding and wash buffers. Aliquots of the various fractions were assayed following SDS/PAGE by immunoblotting using the anti-FLAG M1 antibody for detection (Fig. 5). Comparison of the immunoreactivity in lanes 1 and 2 suggested that >90% of the receptor bound to the M1-agarose matrix, and the strength of the signals in the elution fraction suggested that the recovery of receptor was approximately 30%. Assessment of the purification factor at this stage was difficult due to the very low levels of protein in the M1-agarose eluted fractions; however, employment of the M1-agarose chromatography at earlier stages in the purification scheme indicated that a minimal 50-fold purification was achieved routinely.

3.4. Mass spectrometry

The eluted fractions from the M1 antibody agarose matrix were pooled, and the purified epitope-tagged δ receptor was concentrated using a Centricon 30 filter. The purified receptor was resolved on a 12% SDS–PAGE gel, stained with Coomassie blue, and the 65-kDa receptor band was excised and digested with trypsin. The enzymatic digest was desalted and mass spectra were collected on an Applied Biosystems Voyager MALDI DE-PRO and an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics. A portion of the mass spectrum is shown in Fig. 6. For interpretation of the mass spectra, theoretical trypsin cleavage of the FLAG- and His-tagged δ opioid receptor was performed using the MS-Digest program available at the University of California, San Francisco (prospector.ucsf.edu). The mass of peaks recorded in the mass spectra were matched within 0.3 Da or better to the theoretical masses of δ opioid receptor tryptic peptides.

Using the FindMod tool from the Expasy Web site (www.expasy.org), the mass spectrum was searched for unmodified receptor fragments. The molecular masses of four prominent tryptic peptides in the sample spectrum (with masses of 2580.36, 1853.98, 1198.57, and 999.45) matched the theoretical masses of peptides derived from the carboxyl terminal region of the epitope-tagged δ receptor (Fig. 6 and Table 2). In addition, nine other tryptic peptides derived from transmembrane domain 2, intracellular loops 2 and 3, and the carboxyl terminal domain of the δ opioid receptor were identified in the mass spectrum (Table 2).

For further confirmation of the identity of δ opioid receptor peptide fragments, several tryptic peptides were analyzed by mass spectrometry in precursor selective ms/ms mode. The ms/ms spectrum and fragmentation pattern of the 1854 Da peptide, FEGKPIPNPLLGLDSTR, derived from the C-terminal domain of the tagged δ opioid receptor is displayed in Fig. 7. The fragmentation pattern displayed extensive coverage of the parent peptide, and the daughter

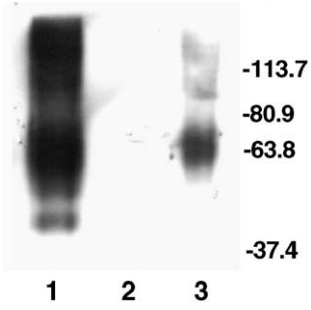

Fig. 5. Purification of the δ opioid receptor using immunoaffinity chromatography. Eluates from the Ni-NTA chromatography were pooled and incubated batch-wise with 1 ml of the M1-agarose beads for 18 h at 4 °C with gentle mixing. The receptor/M1 bead suspension was transferred to a chromatography column, the flow through was collected, the column was washed with 30 volumes of 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% glycerol, and then eluted with 0.05% dodecyl maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, and 5% glycerol. Aliquots of the various fractions were assayed by Western blotting using the anti-FLAG M1 antibody for detection. Lane 1: 0.5% of the input to the M1 column; lane 2: 0.5% of the flow through fraction; lane 3: 0.5% of the M1 eluate.

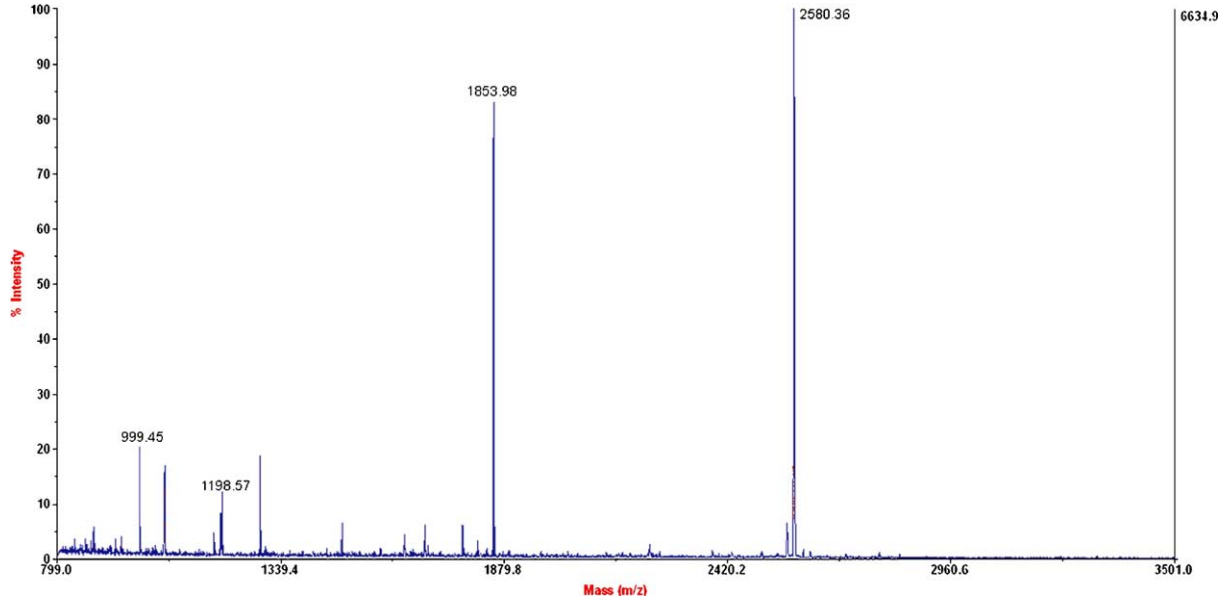

Fig. 6. Mass spectrum of the tryptic digest derived from the purified δ opioid receptor. The mass spectrum was collected on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics. A portion of the mass spectrum derived from tryptic fragments of the 65 kDa band is displayed. For interpretation of the mass spectra, a theoretical trypsin cleavage of the epitope-tagged δ opioid receptor was performed using the MS-Digest program available at the University of California, San Francisco (prospector.ucsf.edu). The masses of the peaks recorded in the mass spectra were matched within 0.3 Da or better to the theoretical masses of δ opioid receptor tryptic peptides, and four of the peaks corresponding to peptides derived from the δ opioid receptor are labeled.

fragments were consistent with the amino acid sequence of the parental peptide.

The tryptic peptides that were detected and identified by mass spectrometry constitute coverage of 28% of the epitope-tagged δ opioid receptor amino acid sequence, corresponding to 117 amino acids out of 425 total amino acids (Fig. 8).

4. Discussion

In this study, a FLAG-tagged δ opioid receptor has been engineered to contain a hexahistidine tag at its carboxyl terminus to facilitate purification of the receptor. The epitope-tagged δ opioid receptor was expressed at relatively high levels in transfected HEK 293 cell lines, and the receptor engaged opioid ligands with expected high affinity. Evidence was obtained that the modified receptor was functional and capable of signal transduction, in that chronic agonist treatment resulted in downregulation of the receptor.

The δ opioid receptor was extracted from transfected HEK 293 cell membranes with *n*-dodecyl-β-D-maltoside in an active form and a procedure for purifying the receptor was developed that employed a combination of chromatographic methods and SDS/PAGE. Following digestion of the purified receptor with trypsin, MALDI-TOF and

Table 2
Peptides identified in enzymatic digests of the purified δ opioid receptor by mass spectrometry

Observed mass (Da)	Predicted mass (Da)	Amino acid (AA) sequence	AA number, location
2855.53	2855.52	TATNIYIFNLALADALATSTLPFQSAK	90–116, TM2
1029.53	1029.56	YIAVCHPVK	155–163, ICL2
1203.68	1203.71	SVRLLSGSKEK	250–260, ICL3
903.26	903.50	EKDRSLR	259–265, ICL3
901.24	901.57	SLRRITR	263–269, ICL3
1195.66	1195.67	QEPGSLRRPR	348–357, C-term
1271.09	1270.71	RPRQATTRER	355–364, C-term
861.06	861.45	QATTRER	358–364, C-term
1198.57	1198.56	GNSADIQHSGGR	382–393, C-term
2580.36	2580.38	SSLEGPRFEGKPIPNPLLGLDSTR	394–417, C-term
1853.98	1854.01	FEGKPIPNPLLGLDSTR	401–417, C-term
2834.40	2834.43	FEGKPIPNPLLGLDSTRTGHHHHHH	401–425, C-term
999.45	999.44	TGHHHHHH	417–425, C-term

Epitope-tagged receptor location: ICL, intracellular loop; TM, transmembrane domain; C-term, carboxyl-terminal domain. Note that the numbering system used accounts for the presence of epitope tags at the N- and C-termini; therefore, the amino acid numbers will differ from those found in the literature for the unmodified δ opioid receptor. The C-terminus of the unmodified receptor is Ala 380.

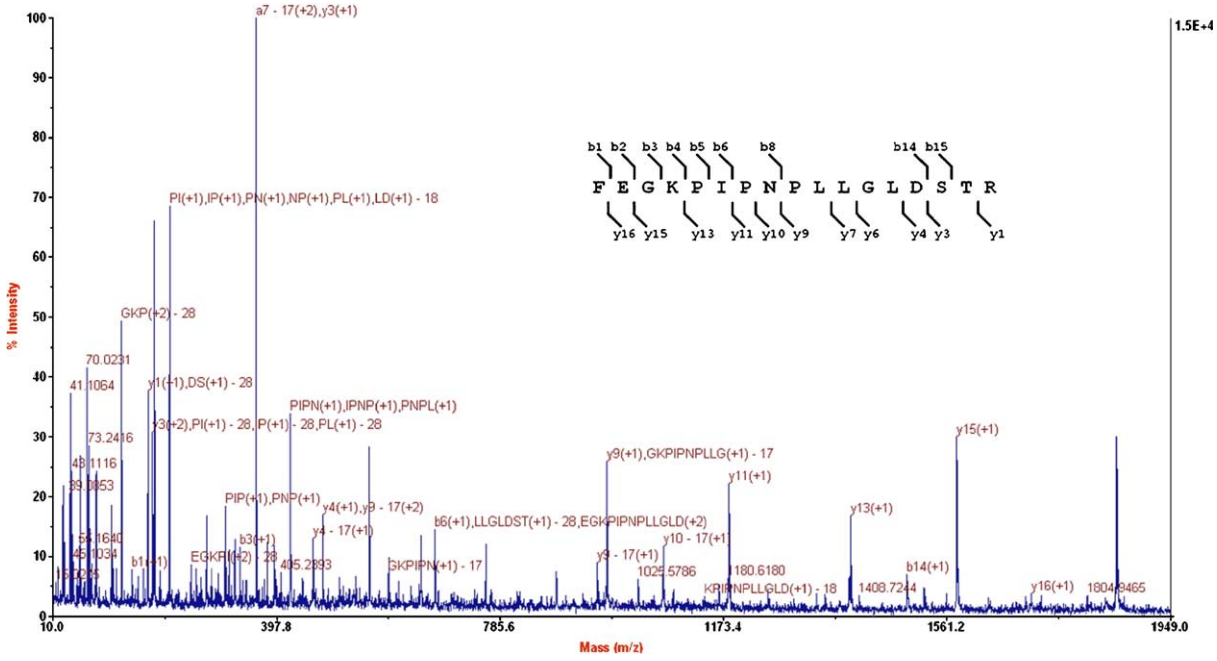

Fig. 7. Precursor selective ms/ms mass spectrum of the 1854 Da tryptic peptide derived from the purified δ opioid receptor. The ms/ms spectrum and fragmentation pattern of the 1854 Da peptide, FEGKPIPNPLLGLDSTR, derived from the carboxyl terminus of the epitope-tagged δ opioid receptor is shown. The fragmentation pattern contained extensive coverage of the parent peptide and confirmed its amino acid sequence. The fragmentation pattern of the parent peptide and observed daughter ions are displayed in the inset.

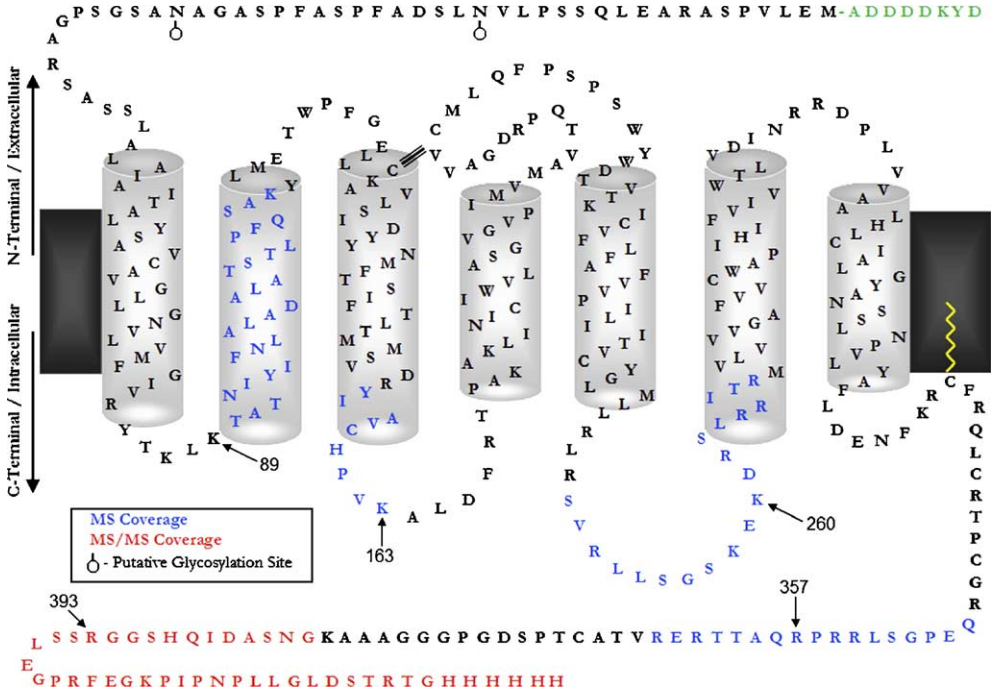

Fig. 8. Amino acid sequence and putative transmembrane topology of the epitope-tagged δ opioid receptor. The FLAG epitope at the extracellular N-terminus is shown in green; the hexahistidine tag is located at the intracellular carboxyl terminus. Dark rectangles depict the plasma membrane. Sites of potential N-linked glycosylation in the N-terminal domain are indicated by circles. The putative disulfide bond between extracellular cysteines is indicated between the second and third extracellular loops, and a putative site of palmitoylation is depicted in yellow on the relevant cysteine in the carboxyl terminal domain. Tryptic peptides identified by MS and MS/MS mass spectrometry are shown in blue and red, respectively. Note that the numbering system used for the epitope-tagged receptor will differ from the unmodified δ opioid receptor found in the literature due to the presence of the 8-amino acid FLAG epitope at the N-terminus and the 45-amino acid extension at the C-terminus consisting of the V5 epitope, linker amino acids, and the hexahistidine tag. The C-terminus of the unmodified receptor is Ala 380.

precursor-selective ms/ms mass spectrometry were used to identify and characterize peptides derived from the δ opioid receptor. To our knowledge, this study is the first to describe analysis of a purified δ opioid receptor using mass spectrometry.

To date, this laboratory has not been successful in purifying wild-type μ, δ, or κ opioid receptors by immunoprecipitation using commercially available antibodies to native opioid receptors. In order to achieve the necessary purification, it was assumed that it would be critical to engineer the δ opioid receptor to contain peptide tags that would permit the use of immobilized metal affinity chromatography and immunoaffinity chromatography. Opioid receptors containing the FLAG epitope at the amino-terminus of the receptor have been studied extensively [7,8,14,27,30]. Since the FLAG epitope at the δ receptor amino-terminus was tolerated well and did not interfere with normal receptor function, this construct was used as the starting point for insertion of a hexahistidine tag. It was observed that δ opioid receptors bearing the hexahistidine tag at the carboxyl terminus were expressed in HEK 293 cells at five- to ten-fold higher levels than receptors with hexahistidine tags following the FLAG epitope at the amino terminus; hence, amino-terminal FLAG-tagged and carboxyl terminal hexahistidine-tagged receptor constructs were chosen for further investigation.

Based on the relatively high level of expression in transfected HEK 293 cell lines (10 pmol/mg protein), we reasoned that it might be feasible to purify opioid receptors using this system. The receptor was solubilized from cell membranes in an active form with a B_{max} of 1.2 pmol/mg protein; at this level of expression, one would theoretically have to purify the 46-kDa receptor approximately 18,000-fold in order to achieve homogeneity. Due to the lability of ligand binding activity, the solubilized receptor was detected by immunoblot analysis during later stages of the purification procedure. This thwarted attempts to quantify receptor purification and yield at each chromatographic step accurately and to calculate the specific activity of the purified receptor. However, by comparing the signal strength of the receptor immunoreactivity on Western blots before and after each chromatographic procedure, we obtained estimates for purification factors and yield at each step. Based on these approximations, we estimate that the receptor was purified 18,000-fold at an overall yield of 10%.

By employing trypsin cleavage, we achieved 28% coverage of the δ opioid receptor using mass spectrometry. The peptides identified by mass spectrometry were derived from transmembrane domain 2, the second and third intracellular domains, and the medial and distal carboxyl terminal domain. We have shown previously that the δ opioid receptor expressed in HEK 293 cells is an N-linked glycoprotein [7], and the primary amino acid sequence contains two consensus sites for N-linked glycosylation in the amino terminal domain. As a result, it was expected and confirmed that unmodified tryptic peptides derived from a large portion of the amino terminal domain would not be detected. In a similar manner, other posttranslational modifications of the receptor, such as phosphorylation, methylation, acetylation, palmitoylation, and ubiquitination would alter the predicted masses of peptides in the enzymatic digests; hence, they would not be detected with the computer search for unmodified receptor peptides used in this study. The development of a viable procedure for purifying the δ opioid receptor from mammalian cells as described in this report should facilitate detailed analyses of posttranslational modifications of the receptor, such as glycosylation, palmitoylation, and phosphorylation, under basal and agonist-stimulated conditions using mass spectrometry.

Acknowledgments

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