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Research report

Purification and mass spectrometric analysis of the μ opioid receptor

Keith H. Christoffers[a], Hong Li[b], Susan M. Keenan[c], Richard D. Howells[a,b,*]

[a] Department of Neuroscience, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA
[b] Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA
[c] Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA

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Abstract

A mouse μ 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 pmol/mg protein. ^{3}[H]Bremazocine exhibited high affinity binding to the epitope-tagged μ opioid receptor with a K_D of 1.0 nM. The agonists [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), morphine and [D-Ala^2,D-Leu^5]enkephalin (DADL) competitively inhibited bremazocine binding to the tagged μ receptor with K_I's of 3.5, 17 and 70 nM, respectively. Chronic treatment of cells expressing the epitope-tagged μ receptor with DAMGO resulted in down-regulation of the receptor, indicating that the tagged receptor retained the capacity to mediate signal transduction. The μ 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 wheat germ agglutinin (WGA)–agarose chromatography, Sephacryl S300 gel filtration chromatography, immobilized metal affinity chromatography, immunoaffinity chromatography, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) permitted purification of the receptor. The purified μ opioid receptor was a glycoprotein that migrated on SDS/PAGE with an apparent molecular mass of 80 kDa. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was used to identify and characterize peptides derived from the μ opioid receptor following in-gel digestion with trypsin or chymotrypsin, and precursor-derived tandem mass spectrometry (ms/ms) confirmed the identity of several peptides derived from enzymatic digestion of the μ opioid receptor.

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

Keywords: FLAG epitope; Human embryonic kidney; Nickel-nitrilotriacetic acid

1. Introduction

There are three types of opioid receptors, designated μ, δ and κ, and they are members of the G protein-coupled receptor superfamily. Opioid receptors mediate the effects of endogenous opioid peptides in the central, peripheral and enteric nervous systems. The μ opioid receptor is also the molecular target of opioid drugs, such as heroin, morphine, fentanyl and methadone [7]. Knockout mice lacking a functional μ opioid receptor do not display analgesia, tolerance or physical dependence to opioid drugs [21]. 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 opioid receptor activation results in the coupling to G proteins on the intracellular aspect of the plasma membrane. The activated G proteins regulate a variety of effectors, including adenylyl cyclase, MAP kinase, phosphatidylinositol 3-kinase, K^+ channels and Ca^{2+} channels [3,4,17,26,27].

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

* Corresponding author. Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, USA. Tel.: +1-973-972-5652; fax: +1-973-972-5594.
E-mail address: howells@umdnj.edu (R.D. Howells).

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Chronic use of opioid drugs leads inevitably to physical dependence and a state of tolerance, in which the pharmacological efficacies of agonists are attenuated. Physical dependence becomes evident in humans when prolonged opioid drug use is terminated, resulting in withdrawal symptoms lasting from several days to weeks [14]. 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 [16,22].

Short-term exposure of opioid receptors to agonists leads to an adaptive response to acute effects that is referred to as receptor desensitization, while chronic exposure leads to receptor down-regulation, which involves proteolytic degradation of the receptors [18,25]. It is probable that agonist-induced down-regulation of opioid receptors contributes to opioid tolerance. Homologous desensitization is initiated by phosphorylation of the agonist-activated G protein-coupled receptor by members of the G protein-coupled receptor kinase family, and subsequent binding of arrestin proteins, which effectively uncouple the interaction of activated G protein-coupled receptors from heterotrimeric G proteins. In addition to preventing receptor/G protein interactions, arrestins stimulate internalization by direct interactions with two proteins of the endocytic machinery, clathrin and AP-2 [9,12]. It has been shown that μ and δ opioid receptors are phosphorylated by G protein-coupled receptor kinases following agonist treatment [23,32], 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 [30,32]. Morphine analgesia is enhanced in knockout mice lacking β-arrestin 2, pointing to a role for β-arrestin 2 in desensitization of the μ 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.

The mechanism for G protein-coupled receptor proteolysis has been generally assumed to involve internalization and trafficking from endosomes to lysosomes, however, we provided evidence that the ubiquitin/proteasome pathway plays a prominent role in agonist-induced opioid receptor down-regulation [6]. Proteasome inhibitors blocked agonist-induced down-regulation of μ and δ opioid receptors, while inhibitors of calpain, caspases and lysosomal cathepsins had no effect. We also provided evidence that μ and δ opioid receptors are ubiquitinated, which serves to target protein substrates to the proteasome complex. Down-regulation of the human κ opioid receptor has also been shown to be partially attenuated by proteasome inhibitors [19]. 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 [24]. 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 [28], the CXCR4 chemokine receptor [10,20] and the CCR5 chemokine receptor [10].

In this study, we report the solubilization and purification of the μ opioid receptor expressed in human embryonic kidney (HEK) 293 cells, and the identification and characterization of peptides derived from proteolytic digestion of the purified receptor using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and tandem mass spectrometry (ms/ms).

2. Materials and methods

2.1. Construction of hexahistidine-tagged opioid receptors

A plasmid encoding the μ opioid receptor with a FLAG epitope at the N-terminus (provided by Dr. Mark von Zastrow, UCSF) was used as a polymerase chain reaction (PCR) template to generate a receptor that also contained a hexahistidine tag at its C-terminus. Oligonucleotide primers employed for the PCR were 5′-μ Flag, 5′-GCC ATG AAG ACG ATC ATC GCC, which was the sense primer used to amplify the 5′-end of the Flag-tagged μ opioid receptor, and 3′-MOR no stop, 5′-GGG CAA TGG AGC AGT TTC TGC, an antisense primer that deletes the termination codon from the 3′-end of the μ 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 μ 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 μ 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 Core 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 units/ml penicillin and 100 μg/ml streptomycin sulfate. HEK 293 cells were transfected with the receptor expression plasmid using Lipofectamine (Invitro-

gen). Cells expressing the epitope-tagged μ 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 μ 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 35,000 × *g* for 20 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 soaked 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 non-linear regression using Prism 3.0c (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 μ receptor by immunoblotting, samples were heated at 40 °C for 5 min in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gel loading buffer (BioRad), resolved by SDS/PAGE using 12% gels, then transferred to Immobilon P^{SQ} polyvinylidene difluoride (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% thimerosol, followed by overnight incubation at 4 °C with mouse anti-FLAG M1 monoclonal antibody (Sigma). Membranes were then washed and incubated with 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 [13]. Briefly, soluble receptors were incubated in duplicate 0.25 ml aliquots for 1 h at 0 °C with [^{3}H]bremazocine (0.1–10 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. Wheat germ agglutinin (WGA)–agarose chromatography

All chromatographic procedures were performed in a cold room. The solubilized FLAG- and His-tagged μ receptor (85 ml) was 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 flow through was collected, the resin was washed with 80 ml of buffer containing 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 μ receptor was eluted in 15 ml of wash buffer containing 0.5 M *N*-acetylglucosamine.

2.7. Gel filtration chromatography

Gel filtration chromatography was performed using Sephacryl S300 HR resin (Pharmacia) packed in a 2.6 × 90-cm column. The WGA eluate was diluted to 20 ml and 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 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). Ten milligrams of each protein was dissolved in 20 ml solubilization buffer and subjected to Sephacryl S300 chromatography using the conditions described above. Elution position of the protein standards was monitored by absorbance at 280 nm. A standard curve was generated by plotting the ratio of the elution volume for each protein and void volume of the column versus the logarithm of the protein's molecular weight.

2.8. Immobilized metal affinity chromatography

Fractions containing the μ receptor from Sephacryl S300 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, the flow through fraction was collected, and the matrix was washed with 150 ml of buffer containing 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 was washed sequentially with the same buffer containing 10 mM imidazole, then 20 mM imidazole, and the epitope-tagged μ receptor was eluted with buffer containing 250 mM imidazole.

2.9. Immunoaffinity chromatography

The μ 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 flow through was collected, the column was rinsed with 30 volumes of 0.05% dodecyl maltoside, 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 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.

2.10. SDS/PAGE

Fractions containing the purified 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 80-kDa 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 and chymotrypsin

The gel fragment was diced into 1 mm^3 fragments and washed three 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 and dehydrated as described above. The reduced and alkylated gel slices were rehydrated in 20 μl of 50 mM ammonium bicarbonate, pH 8.3, containing 200 ng of trypsin or chymotrypsin (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 and chymotryptic 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 μ opioid receptor and analysis of ligand affinities

It was found that μ 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 9.8 pmol/mg protein. [^{3}H]Bremazocine exhibited high affinity binding to the epitope-tagged receptor with an apparent dissociation constant (K_D) of 1.0 nM (Fig. 1A). In contrast, receptor constructs containing the hexahistidine tag adjacent to the FLAG epitope at the amino terminus were expressed at 5- to 10-fold lower levels in HEK 293 cells (data not shown).

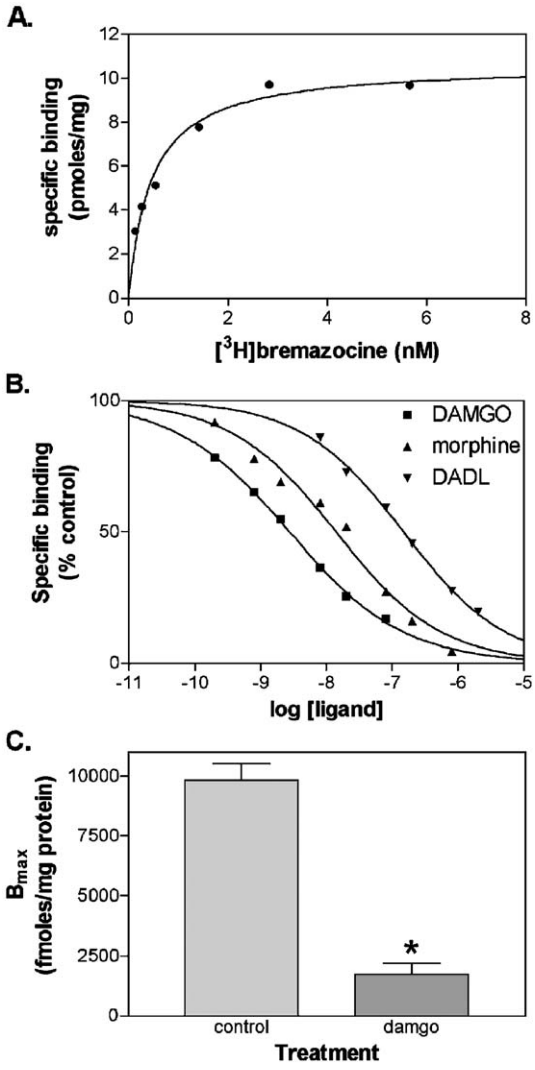

Fig. 1. Characterization of the epitope-tagged μ opioid receptor. (A) Saturation curve of [^{3}H]bremazocine binding. Membrane preparations derived from HEK 293 cells stably expressing the FLAG- and His-tagged μ opioid receptor were incubated with various concentrations of [^{3}H]bremazocine in the presence 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 $K_D = 0.96 \pm 0.14$ nM and a $B_{max} = 9.8 \pm 0.7$ pmol/mg protein. (B) Competition analysis of ligand binding to the epitope-tagged μ opioid receptor. Membrane aliquots were incubated with 1.2 nM [^{3}H]bremazocine in the presence and absence of varying concentrations of DAMGO, morphine and DADL for 60 min at 0 °C. Results from five experiments yielded K_I's of 3.5 ± 1.4, 16.6 ± 4.1 and 69.6 ± 16 nM for DAMGO, morphine and DADL, respectively. (C) Agonist-induced down regulation of the epitope-tagged μ receptor. HEK 293 cells expressing the epitope-tagged μ receptor were incubated at 37 °C in serum-free medium for 18 h in the presence and absence of 1 μM DAMGO. Washed membrane preparations were used for saturation analysis using [^{3}H]bremazocine. B_{max} values for control and DAMGO-treated samples were 8.9 ± 0.8 and 1.7 ± 0.4 pmol/mg protein, respectively ($n = 3$).

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 ligands were determined by competition analysis. The agonists [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), morphine and [D-Ala^2,D-Leu^5]enkephalin (DADL) competitively inhibited bremazocine binding to the engineered μ receptor with K_I's of 3.5, 17 and 70 nM, respectively (Fig. 1B). These values are in excellent agreement with the affinities of these compounds for the wild type μ receptor published previously by this laboratory [5].

3.2. Agonist-induced down-regulation of the epitope-tagged μ opioid receptor

Confirmation that the epitope-tagged μ receptor retained the capacity to be activated and transduce signals was obtained by studying agonist-induced down-regulation of the receptor. Incubation of the HEK 293 cell line for 18 h with 1 μM DAMGO resulted in a significant decrease in the steady state level of receptor (Fig. 1C). Saturation analysis revealed that chronic exposure to DAMGO induced an 80% decrease in B_{max} relative to untreated controls, with little change in the affinity of bremazocine for the receptor (K_D's were 0.9 ± 0.2 and 1.3 ± 0.1 nM for control and DAMGO-treated samples, respectively). The magnitude of the DAMGO-induced down-regulation was very similar to that previously reported by this laboratory [6], indicating that the presence of the hexahistidine tag at the C-terminus of the μ receptor did not interfere with agonist-induced receptor activation and signal transduction.

3.3. Receptor purification

It was necessary to establish which cellular source was optimal for receptor purification before proceeding to large-scale experiments. HEK 293 cells expressing the epitope-tagged μ receptor were harvested from plates, homogenized in 50 mM Tris HCl, pH 7.5, then centrifuged to obtain a crude membrane preparation and the corresponding supernatant. Equal amounts of protein from the homogenate, membrane preparation and supernatant were lysed in SDS/PAGE load-

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

	K_D (nM)	B_{max} (fmol/mg)	% B_{max}
Membrane fraction	1.17 ± 0.05	8540 ± 460	
Soluble fraction	4.06 ± 0.66	2670 ± 180	33 ± 1.5

Membranes from HEK 293 cells expressing the epitope-tagged μ 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, centrifuged at 10,000 × *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 [^{3}H]bremazocine (0.1–10 nM) in the presence and absence of 10 μM cyclazocine. Soluble receptors were precipitated as described previously [13] 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 3.0c. Data are derived from three experiments.

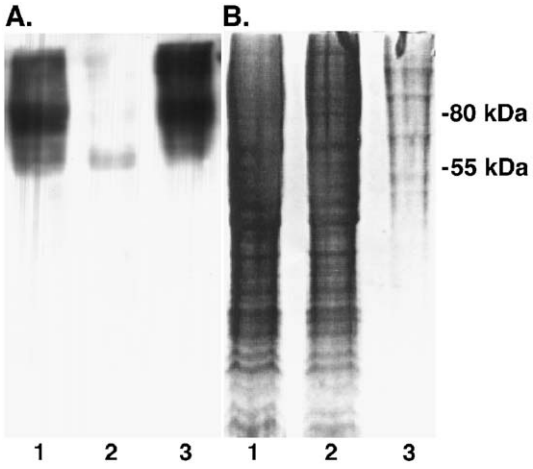

Fig. 2. Purification of the μ opioid receptor using WGA lectin chromatography. The solubilized epitope-tagged μ receptor was incubated batch wise for 18 h at 4 °C with wheat germ agglutinin-agarose beads. The suspension was transferred to a chromatography column, the flow through fraction was collected, 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 μ 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: the solubilized receptor input to the WGA resin (40 μg protein), lane 2: the flow-through fraction (40 μg protein), lane 3: the fraction eluted with *N*-acetylglucosamine (2 μg protein). Protein size markers are indicated to the right of the gels.

ing buffer, and receptor levels were compared by Western blotting. The membrane fraction was enriched in the μ receptor relative to the homogenate, and no receptor immunoreactivity was found in the supernatant (data not shown). As an outcome of this experiment, the decision was made to use washed membrane fractions as the starting source for large-scale receptor solubilization and purification.

The non-ionic detergent, *n*-dodecyl-β-D-maltoside, was employed for solubilization of the μ receptor, based on its ability to solubilize the receptor in an active form and its compatibility with subsequent mass spectrometric analysis of the purified protein. The affinity of bremazocine for the dodecyl maltoside-solubilized μ receptor was 3.5-fold lower than the affinity for the membrane-bound receptor (Table 1). The B_{max} of the soluble preparation was three-fold lower than the B_{max} of the membranes, indicating that the solubilization yield of active receptors was approximately 33%. At an expression level of 2.7 pmol/mg protein, one would have to purify the 49,828-Da receptor approximately 7500-fold to achieve homogeneity.

The stability of the detergent-solubilized μ receptor, as determined by ligand binding, exhibited biphasic kinetics when the soluble ultracentrifugal supernatant was stored at 4 °C. Approximately 70% of the receptor binding inactivated with a half-life of 6 h, while the remaining 30% exhibited a half-life of 90 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.

WGA–agarose chromatography was chosen as the first step in the purification scheme, based on observations made two decades ago that opioid receptors were retained by and could be eluted from WGA lectin columns [11]. We have also provided evidence that μ and δ opioid receptors are glycoproteins when expressed in HEK 293 cells [6]. The

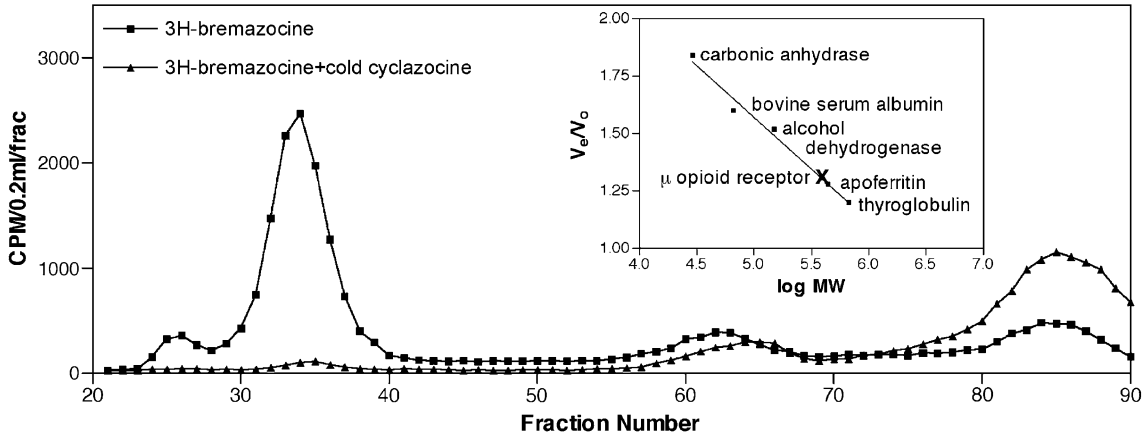

Fig. 3. Gel filtration chromatography of the μ opioid receptor. In this experiment, the *N*-acetylglucosamine eluate from a WGA-agarose column was divided in two and incubated with 2 nM [^{3}H]bremazocine in the absence and presence of 1 μM cyclazocine for 1 h at 0 °C, and the two samples were applied sequentially to a 2.6 × 90 cm Sephacryl S300 column 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 25–26 (not shown) and the major peak of the ligand/receptor complex eluted in fractions 32–38. Prelabeling of the receptor in the presence of a molar excess of unlabeled cyclazocine resulted in total displacement of [^{3}H]bremazocine from the ligand/receptor complex. Inset. Molecular weight estimation of the ligand-occupied μ 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/μ receptor complex was 385,500.

ultracentrifugal supernatant containing the dodecyl maltoside-solubilized μ receptor was fractionated using 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 monoclonal antibody for detection in order to estimate the extent of binding to the WGA resin, the elution yield and the purification factor (Fig. 2A), and the other was stained with Coomassie Blue (Fig. 2B). The theoretical molecular weight of the epitope-tagged μ receptor is 49,828; however, the receptor immunoreactivity following SDS/PAGE presents as two broad bands with apparent molecular masses of approximately 55 and 80 kDa, presumably due to post-translational modifications of the receptor, as suggested previously [6]. The epitope-tagged μ 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). It is interesting to note that the predominant 80-kDa band bound nearly quantitatively to the WGA resin while the 55-kDa immunoreactive species did not. The 55-kDa band of receptor immunoreactivity presumably does not contain the requisite terminal *N*-acetylglucosamine or chitobiose moieties that are required for binding to WGA, however, its characterization will require further study and purification. The amount of protein loaded in lanes 1 and 2 (40 μg) represented 0.01% of the total solubilized receptor preparation and flow through fraction, respectively. The amount of protein loaded in lane 3 (2 μg) represented 0.06% of the eluted fraction. Quantification of the respective immunoreactive bands in Fig. 2A using NIH Image software, version 1.61 indicated that the total immunoreactivity in lane 3 was two-fold greater than the immunoreactivity in lane 1. Accordingly, we estimated that the yield of receptor from the WGA column was approximately 33%. Given that the WGA eluate in lane 3 contained twice the amount of immunoreactive μ opioid receptor with 1/20th the amount of protein, we estimated that the purification factor for the WGA chromatography was approximately 40-fold.

The next step in the purification procedure was gel filtration chromatography, which provided an estimate of the size of the solubilized ligand/receptor complex, as well as purification of the receptor. At this stage, receptor binding was still sufficiently active to follow the receptor complex by prelabeling the WGA–agarose eluate with [^{3}H]bremazocine prior to application to the Sephacryl column. Initially, Sephacryl S200 resin was used; however, it was found that the detergent-solubilized μ receptor eluted coincident with the void volume, which contained the majority of protein (data not shown). Based on these results, Sephacryl S300 resin was employed. With this matrix, the solubilized μ receptor complex was well separated from the protein in the void volume (Fig. 3). Recovery of the prebound receptor was typically 75–85%, with a purification factor of two- to three-fold under these conditions. 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 24–27) and the major radioactive peak representing the bremazocine/receptor complex in fractions 32–38 were completely displaced by the excess unlabeled ligand (Fig. 3), and the radioactivity appeared at the elution position of free bremazocine (fractions 80–90). These data provided strong evidence that the major peak of radioactivity in fractions 32–38 represented the ligand-bound receptor complex. This conclusion was confirmed by Western blotting (see Fig. 4, below).

Calibration of the Sephacryl S300 column with protein standards indicated that the detergent-solubilized μ receptor complex migrated with an apparent molecular mass of approximately 385.5 kDa (Fig. 3, inset). This is in contrast to the apparent molecular mass of the glycosylated receptor immunoreactive band of 80 kDa on SDS/PAGE noted earlier. The theoretical molecular weight of the epitope-tagged μ receptor without post-translational modifications is

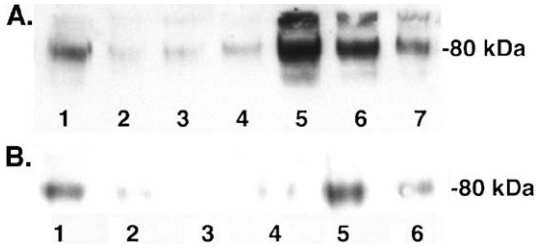

Fig. 4. Purification of the μ opioid receptor using immobilized metal affinity and immunoaffinity chromatography. (A) Fractions containing the bremazocine-labeled receptor from Sephacryl S300 chromatography were pooled and incubated batch wise for 18 h at 4 °C with the Ni-NTA matrix. The receptor/Ni-NTA suspension was applied 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 μ 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: 0.02% of the input to the Ni-NTA column (the pooled Sephacryl S300 fractions); lane 2: 0.02% of the Ni-NTA flow through fraction; lane 3: 0.2% of the 10 mM imidazole wash; lane 4: 0.2% of the 20 mM imidazole wash; lanes 5–7: 0.6% of the first, second and third 250 mM imidazole eluate, respectively. (B) 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: 0.2% of the input to the M1 column (the pooled Ni-NTA eluates); lane 2: 0.4% of the flow through fraction; lane 3: 0.4% of the wash fraction; lanes 4–6: 1% of successive elutions with 10 mM EDTA, respectively.

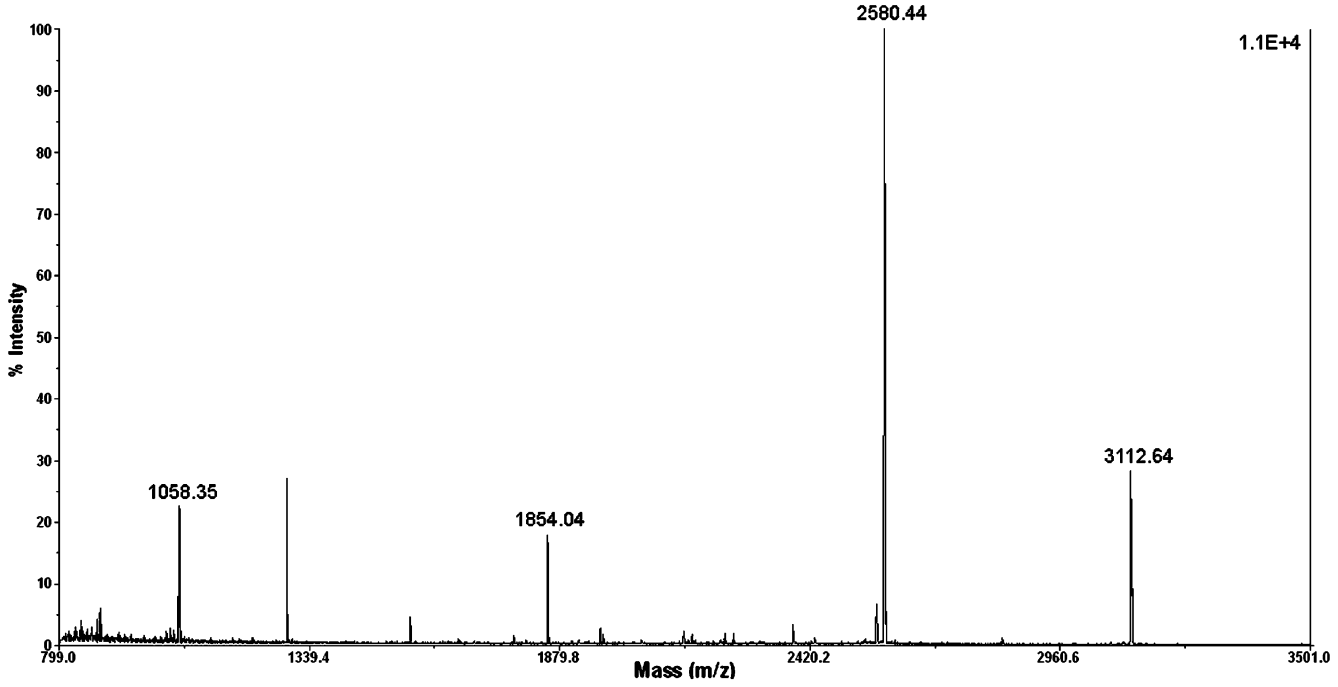

Fig. 5. Mass spectrum of the tryptic digest derived from the purified μ opioid receptor. The mass spectrum was collected on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics. A portion of the mass spectrum derived from tryptic fragments of the 80-kDa band is displayed. For interpretation of the mass spectra, a theoretical trypsin cleavage of the epitope-tagged μ 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 μ opioid receptor tryptic peptides.

49,828. The solubilized receptor is contained within detergent micelles, which for *n*-dodecyl-β-D-maltoside (CMC = 0.12 mM, aggregation number = 78–92) would have an average molecular mass of 40–47 kDa. It is obvious that under gel filtration conditions some degree of protein aggregation is occurring within the detergent

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

Observed mass (Da)	Predicted mass (Da)	Amino acid (AA) sequence	AA number, location*
829.24	829.47	DRNLRR[a]	276–281, ICL3
846.41	846.47	SIVCVVGL[b]	80–87, TM1
855.03	855.39	AFLDENF[b]	341–347, C-term
861.03	861.42	FMATTKY[b]	208–212, ECL2
879.29	879.46	MLSGSKEK[a]	268–275, ICL3
928.23	928.58	NLRRITR[a]	278–284, ICL3
930.24	930.51	EKDRNLR[a]	274–280, ICL3
993.43	993.54	SIVCVVGLF[b]	80–88, TM1
1029.34	1029.55	YIAVCHPVK[a]	170–178, ICL2
1058.35	1058.47	DENFKRCF[b]	344–351, C-term
1144.55	1144.62	KICVFIFAF[b]	237–245, TM5
1191.66	1191.66	VVVAVFIVCW[b]	288–297, TM6
1550.74	1550.83	EGPRFEGKPIPNPL[b]	419–432, C-term
1636.72	1636.77	CPQTGSPSMVTAITIM[b]	61–76, N-term
1697.73	1697.84	ADALATSTLPFQSVNY[b]	117–132, TM2
1741.84	1741.84	LGLDSTRTGHHHHHH[b]	433–447, C-term
1854.04	1854.01	FEGKPIPNPLLGLDSTR[a]	423–439, C-term
1904.96	1904.97	TNHQLENLEAETAPLPK[a]	387–403, C-term
2580.44	2580.38	SSLEGPRFEGKPIPNPLLGLDSTR[a]	416–439, C-term
2834.44	2834.43	FEGKPIPNPLLGLDSTRTGHHHHHH[a]	423–447, C-term
3112.64	3112.53	EHPSTANTVDRTNHQLENLEAETAPLPK[a]	376–403, C-term

[a] Tryptic peptide.
[b] Chymotryptic peptide.
* Receptor location: N-term, amino-terminal domain; ICL, intracellular loop; TM, transmembrane domain; ECL, extracellular loop; C-term, carboxyl-terminal domain.

micelles, involving either multimers of the receptor itself or association with other proteins.

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 monoclonal antibody for detection (Fig. 4A). Comparison of the immunoreactive receptor signal in lanes 1 and 2 (corresponding to the column input and flow-through fractions, respectively) suggested that >80% of the μ receptor bound to the Ni-NTA matrix, and the signals in lanes 5–7 suggested that the majority of the bound receptor was eluted from the column with 250 mM imidazole. The strength of the signal in lane 5 was approximately 10 times the strength of the signal in lane 1, with only 1/5th the amount of protein, suggesting that a purification factor of 50-fold was achieved using immobilized metal affinity chromatography.

Following Ni-NTA chromatography, the μ 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 can be 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. 4B). Comparison of the immunoreactivity in lanes 1 and 2 suggested that >80% of the receptor bound to the M1-agarose matrix, and the strength of the signals in the elution fractions suggested that the recovery of receptor was approximately 50%. Assessment of the purification factor at this stage was difficult due to the low levels of protein in the M1-agarose eluted fractions; however, employment of the M1-agarose chromatography at earlier

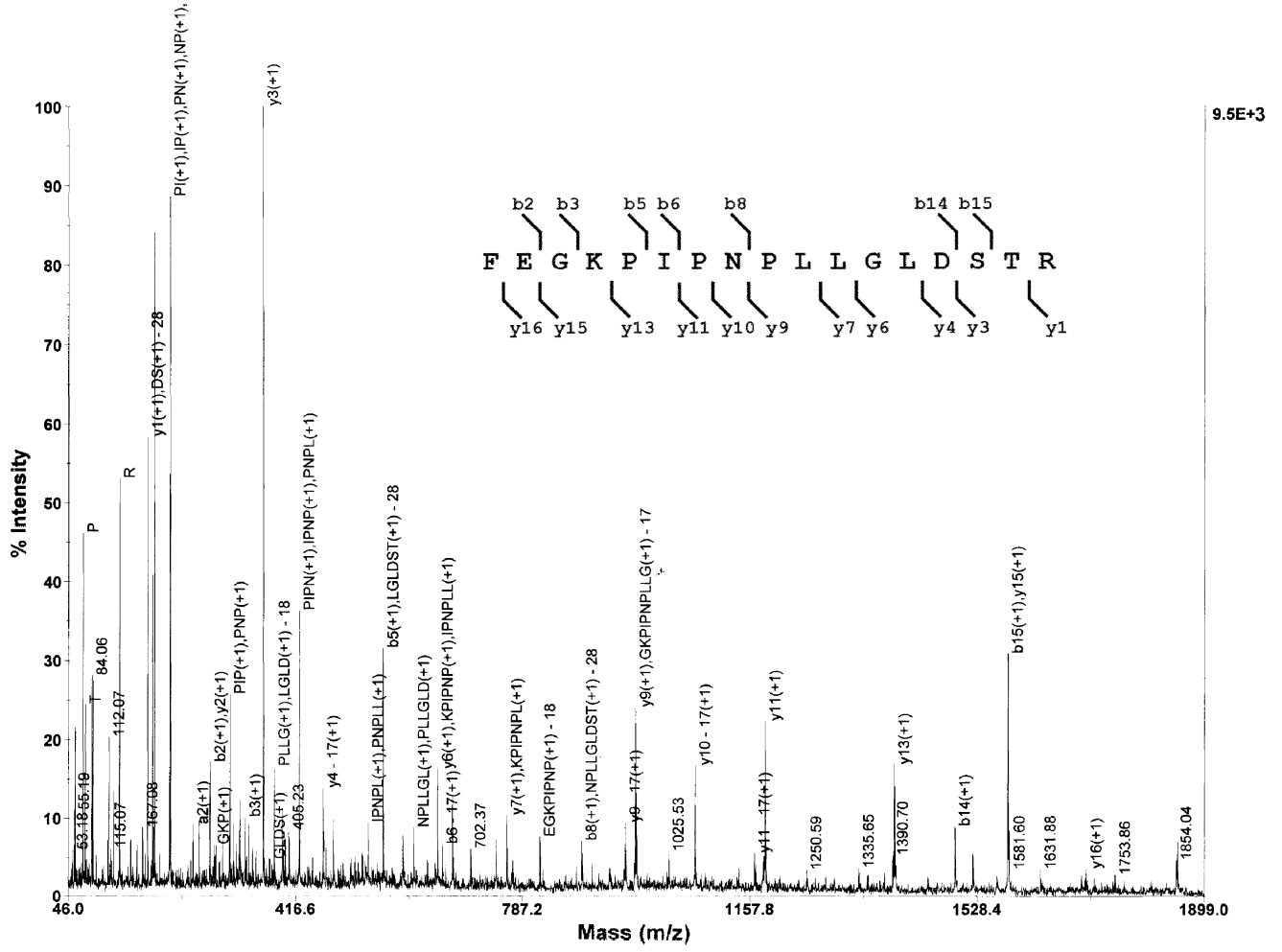

Fig. 6. Precursor selective ms/ms mass spectrum of the 1854-Da tryptic peptide derived from the purified μ opioid receptor. For further confirmation of the identity of μ opioid receptor peptide fragments, several tryptic peptides were analyzed in precursor selective ms/ms mode. Shown above is the ms/ms spectrum and fragmentation pattern of the 1854-Da peptide, FEGKPIPNPLLGLDSTR, derived from the C-terminus of the epitope-tagged μ opioid receptor. 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.

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 μ receptor was concentrated using an Amicon Centricon 30 filter. The purified receptor was resolved on a 12% SDS-PAGE gel, stained with Coomassie blue, and the 80-kDa receptor band was excised with a sterile stainless steel scalpel. The gel fragment was digested in 50 mM ammonium bicarbonate, pH 8.3, containing 200 ng of trypsin or chymotrypsin (Promega, sequencing grade).

The enzymatic digests were desalted using C_{18} ZipTips 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 derived from tryptic fragments of the 80 kDa band is shown in Fig. 5. For interpretation of the mass spectra, theoretical trypsin and chymotrypsin cleavage of the FLAG- and His-tagged μ 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 μ opioid receptor tryptic peptides.

Using the FindMod tool from the Expasy website (http://www.expasy.org), the mass spectrum was searched for unmodified receptor fragments. The molecular masses of two prominent tryptic peptides in the sample spectrum matched the theoretical masses of two peptides (with masses of 2580.38 and 3112.53) derived from the carboxyl-terminal region of the epitope-tagged μ receptor (Table 2). In addition, eight other tryptic peptides derived from intracellular loops 2 and 3 and the carboxyl-terminal domain of the μ opioid receptor were identified in the mass spectrum (Table 2).

For further confirmation of the identity of μ 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 μ opioid receptor is displayed in Fig. 6. The fragmentation pattern displayed extensive coverage of the parent peptide, and the daughter fragments were consistent with the amino acid sequence of the parental peptide. Precursor selective ms/ms spectra were also generated for the 1905-, 2580- and 3112.5-Da tryptic peptides, and all of the fragmentation patterns were consistent with the amino acid sequences of each peptide (data not shown).

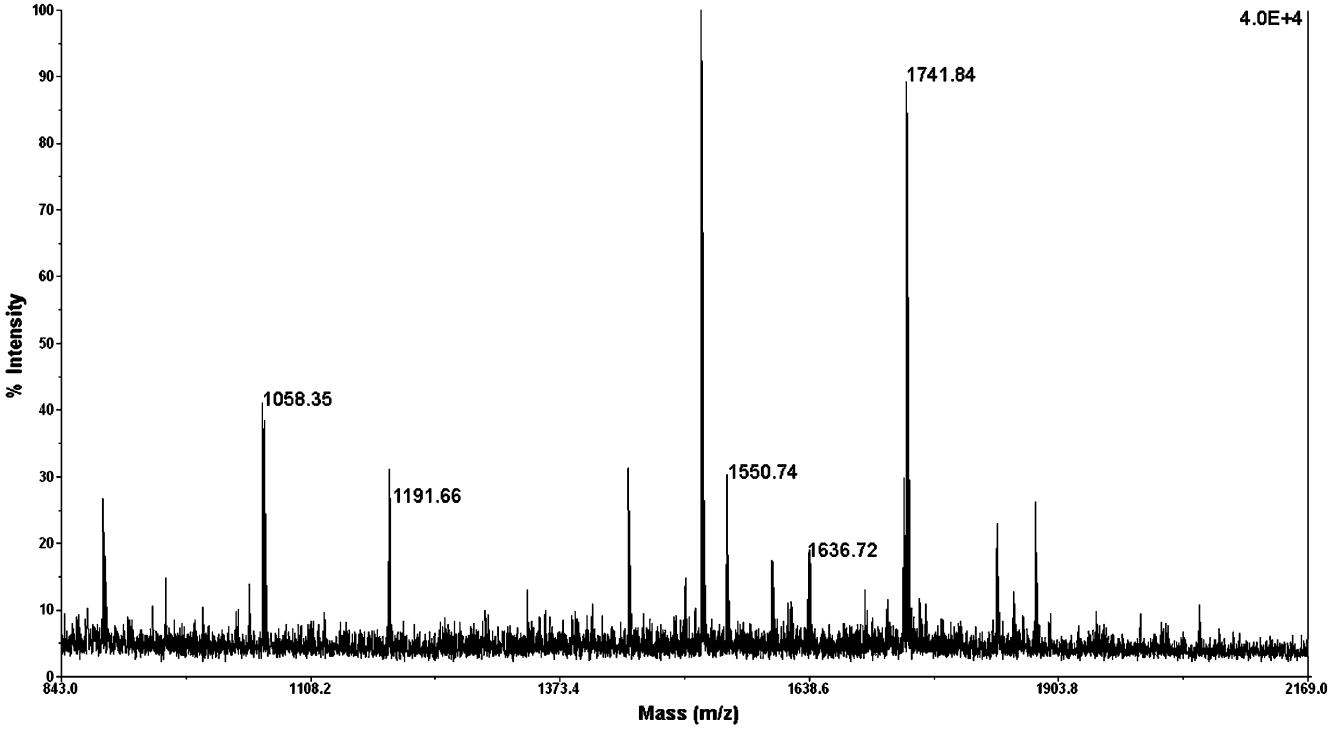

Fig. 7. Mass spectrum of the chymotryptic digest derived from the purified μ opioid receptor. The mass spectrum was collected on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics and analyzed as described in Fig. 5. Some of the receptor-derived chymotryptic peptides are labeled with arrows: the 1058 mass peptide corresponds to DENFKRCF, amino acids 344–351, derived from the proximal C-terminus; the 1192 mass peptide is VVVAVFIVCW, amino acids 288–297, located in transmembrane domain 6; the 1551 mass peptide is EGPRFEGKPIPNPL, amino acids 419–432, derived from the distal C-terminus; the 1637 mass peptide corresponds to CPQTGSPSMVTAITIM, amino acids 61–76, located in the N-terminal domain; and the 1742 mass peptide is LGLDSTRTGHHHHHH, amino acids 433–447, corresponding to the C-terminus of the epitope-tagged receptor.

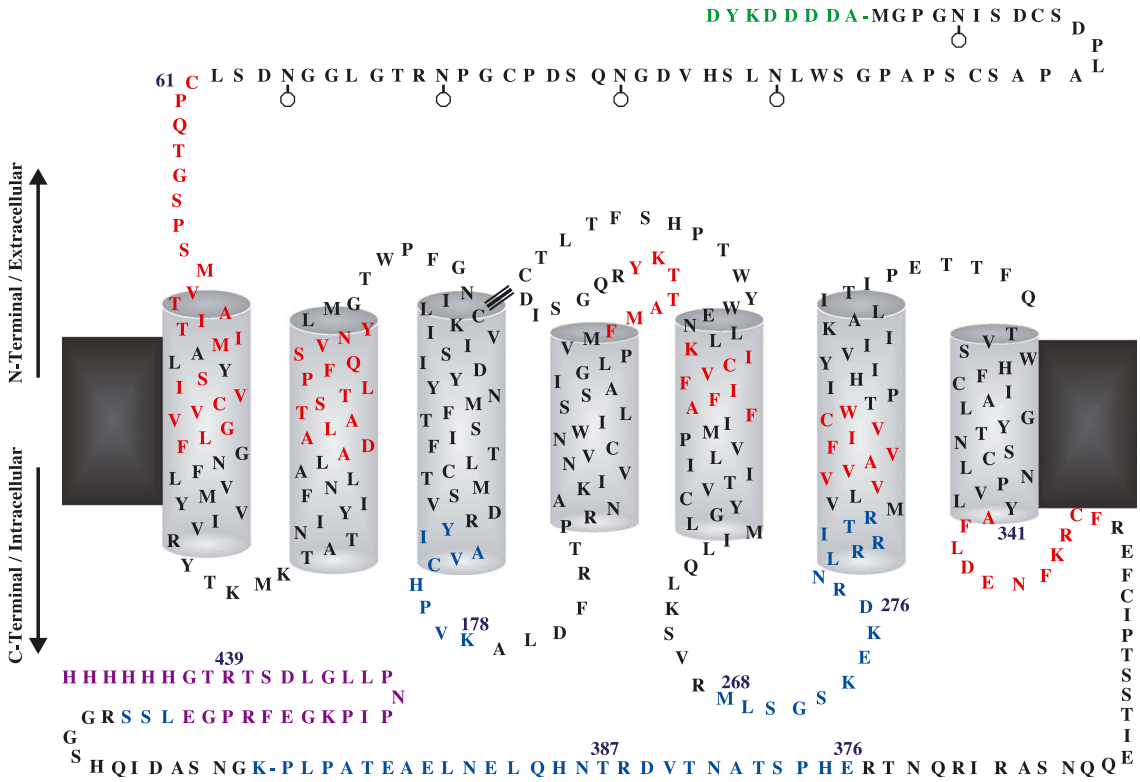

Fig. 8. Amino acid sequence and putative transmembrane topology of the epitope-tagged μ opioid receptor. The FLAG epitope at the extracellular N-terminus is shown in *green*; the hexahistidine tag is located at the intracellular C-terminus. Dark rectangles depict the plasma membrane. Sites of potential *N*-linked glycosylation in the N-terminal domain are indicated by *circles*. The putative disulfide bond between cysteines 144 and 221 is indicated in *dark green*. Tryptic and chymotryptic peptides identified by mass spectrometry are shown in *blue* and *red*, respectively, and overlapping sequences identified following cleavage with both enzymes are indicated in *purple*.

Digestion of the purified μ receptor with chymotrypsin yielded an additional set of 11 unmodified peptides detected by mass spectrometry that matched the theoretical masses of expected μ receptor digestion products (Fig. 7, Table 2). The chymotryptic peptides that were identified with mass spectrometry were widely distributed across the primary structure of the μ receptor. Chymotryptic peptides were derived from the extracellular N-terminal domain, transmembrane domain 1, transmembrane domain 2, the second extracellular loop, transmembrane domain 5, transmembrane domain 6, the proximal C-terminus and the distal C-terminus.

Taken together, the tryptic and chymotryptic peptides that were detected and identified by mass spectrometry constitute coverage of 37% of the epitope-tagged μ opioid receptor amino acid sequence, corresponding to 164 amino acids out of 447 total amino acids (Fig. 8).

4. Discussion

In this study, a FLAG-tagged μ opioid receptor has been engineered to contain a hexahistidine tag at its carboxyl-terminus to facilitate purification of the receptor. The epitope-tagged μ 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 down-regulation of the receptor.

The μ opioid receptor was solubilized with *n*-dodecyl-β-D-maltoside from transfected HEK 293 cell membranes and purified using a combination of chromatographic methods and SDS/PAGE. Following digestion of the purified receptor with trypsin or chymotrypsin, MALDI-TOF and precursor-selective ms/ms mass spectrometry was used to identify and characterize peptides derived from the μ opioid receptor. To our knowledge, this study is the first to describe analysis of a purified opioid receptor using mass spectrometry.

To date, this laboratory has not been successful in purifying wild type μ, δ or κ opioid receptors 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 μ 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 [6,8,15,29,31]. Since the FLAG

epitope at the μ 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 μ opioid receptors bearing the hexahistidine tag at the carboxyl terminus were expressed in HEK 293 cells at 5- to 10-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.

As anticipated, the plasma membrane preparation proved to be the optimal starting source for receptor purification, based on the receptor density in HEK 293 cellular fractions. Assaying the supernatant was of interest due to a recent report of soluble δ opioid receptors in transfected HEK 293 cells [24]. We found no evidence of soluble μ opioid receptors in transfected HEK 293 cells, using radioligand binding assays or immunoblotting for detection.

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 could be solubilized from cell membranes in an active form at 33% yield using the nonionic detergent, *n*-dodecyl-β-D-maltoside. The B_{max} of the initial dodecyl maltoside-solubilized receptor preparation was 2.7 pmol/mg protein. At this level of expression, one would theoretically have to purify the 50-kDa receptor approximately 7500-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. We estimate that the four chromatographic procedures in our purification scheme were capable of the requisite 7500-fold enrichment, with an overall yield of approximately 10%.

By employing a combination of trypsin and chymotrypsin cleavage, we achieved 37% coverage of the μ opioid receptor using mass spectrometry. The peptides identified by mass spectrometry were widely distributed across the primary amino acid sequence of the receptor, and included peptides derived from the distal amino terminus, transmembrane domains 1, 2, 4 and 5, the second extracellular domain, the second and third intracellular domains, and the proximal, medial and distal carboxyl terminal domains.

We have shown previously that the μ opioid receptor expressed in HEK 293 cells is an *N*-linked glycoprotein [6], and the primary amino acid sequence contains five consensus sites for *N*-linked glycosylation in the amino terminal domain (Fig. 8). As a result, it was expected and confirmed that unmodified tryptic and chymotryptic peptides derived from a large portion of the amino terminal domain would not be detected. In a similar manner, other post-translational 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. Detailed analyses of post-translational modifications of the μ opioid receptor under basal and agonist-stimulated conditions using mass spectrometry will be reported elsewhere.

Acknowledgements

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