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Research Report

Purification and mass spectrometric analysis of the κ opioid receptor

Kenneth M. Wannemacher[a], *Alexandra Terskiy*[a,b], *Shengjie Bian*[b,c], *Prem N. Yadav*[b], *Hong Li*[a,b,c], *Richard D. Howells*[a,b,*]

[a]*Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey-Graduate School of Biomedical Science, Newark, NJ, USA*

[b]*Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ, USA*

[c]*Center for Advanced Proteomics Research, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ, USA*

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ABSTRACT

A clonal human embryonic kidney (HEK) 293 cell line was established that stably expressed the rat κ-opioid receptor (rKOR) with a FLAG epitope at the amino terminus. The K_d for [^{3}H] diprenorphine was 1.1±0.2 nM, and the B_{max} was 2.6±0.4 pmol/mg. Dynorphin A (1–13), U69,593 and naloxone competitively inhibited [^{3}H]diprenorphine binding with K_i values of 2.0, 18 and 18 nM, respectively, in good agreement with previously reported affinities for the unmodified receptor. U69,593 stimulated [^{35}S]GTPγS binding in a concentration-dependent manner and caused phosphorylation of mitogen-activated protein (MAP) kinase, indicating that the activated epitope-tagged receptor triggered appropriate signaling pathways. Immunoblot analysis demonstrated that two immunoreactive receptor species with apparent molecular masses of 42 and 52 kDa were expressed. Previous studies indicated that the 42 kDa protein was localized intracellularly and was a precursor of the 52 kDa receptor, which was present at the cell surface. rKOR was extracted from transfected HEK 293 cell membranes with *n*-dodecyl-β-D-maltopyranoside. Sequential use of wheat germ agglutinin chromatography, Sephacryl S300 gel filtration chromatography, anti-FLAG immunoaffinity chromatography and SDS/PAGE permitted purification of the 52 kDa receptor. MALDI-TOF mass spectrometry was used to identify peptides derived from rKOR following sequential in-gel digestion with trypsin and cyanogen bromide. Eighteen rKOR peptides were detected, corresponding to 27.1% coverage of the receptor. Precursor-selective MS/MS confirmed the identity of most of these peptides. In addition, we have identified heat shock protein 70 (HSP70) as a rKOR-interacting protein.

* *Corresponding author*. Department of Biochemistry and Molecular Biology, MSB E643, Newark, NJ 07101-1709, USA. Fax: +1 973 972 5594.
E-mail address: howells@umdnj.edu (R.D. Howells).

Abbreviations: DDM, *n*-dodecyl-β-D-maltopyranoside; HEK, human embryonic kidney; HSP70, heat shock protein 70; MALDI, matrix-assisted laser desorption ionization; MAP, mitogen-activated protein; MS, mass spectrometry; rKOR, rat kappa opioid receptor; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; TOF, time of flight; U69,593, (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide; WGA, wheat germ agglutinin

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1. Introduction

Endogenous opioid peptides and exogenous opiates regulate a multitude of biological processes such as nociception, mood, respiration and gastrointestinal motility (Jaffe and Martin, 1990). The receptor family responsible for mediating these effects was detected in rat brain homogenate by three groups in 1973 (Simon et al., 1973, Pert and Snyder, 1973, Terenius, 1973). Three opioid receptor types have been cloned, designated μ (Chen et al., 1993, Thompson et al., 1993), δ (Evans et al., 1992, Kieffer et al., 1992) and κ (Yasuda et al., 1993), and the receptors are members of the G-protein coupled receptor (GPCR) superfamily. Amino acid sequence alignments indicated that the opioid receptors are approximately 60% identical, with the highest degree of homology in the intracellular loops and transmembrane domains (Chaturvedi et al., 2001a). Opioid agonist-mediated effects are the result of receptor activation and $G_{i/o}$ protein coupling, followed by modulation of effectors such as adenylyl cyclase, mitogen-activated protein (MAP) kinase, phosphatidylinositol-3-kinase, Ca^{2+} channels and K^+ channels (Law et al., 2000).

Activation of KOR produces analgesia, dysphoria, diuresis and anti-pruritic activity (reviewed in Liu-Chen, 2004). KOR agonists have also been shown to be effective in several models of visceral pain (Riviere, 2004). The development of peripherally-acting KOR agonists that lack the dysphoric properties associated with CNS-acting KOR agonists would be of great therapeutic value. KOR agonists may also be useful in alleviating craving in cocaine addicts (Shippenberg et al., 2001) and may be beneficial as anticonvulsive drugs (Loacker et al., 2007). Alternatively, KOR antagonists that are capable of blocking the dysphoria associated with stress and activation of the dynorphin/KOR system may prove useful in a variety of disease states (Land et al., 2008). Further insight into understanding KOR function at the molecular level should promote the feasibility of targeting KOR for therapeutic purposes.

Kappa opioid receptors are regulated by both acute and chronic agonist treatment. Following agonist binding and G-protein activation, KOR is rapidly phosphorylated by G-protein

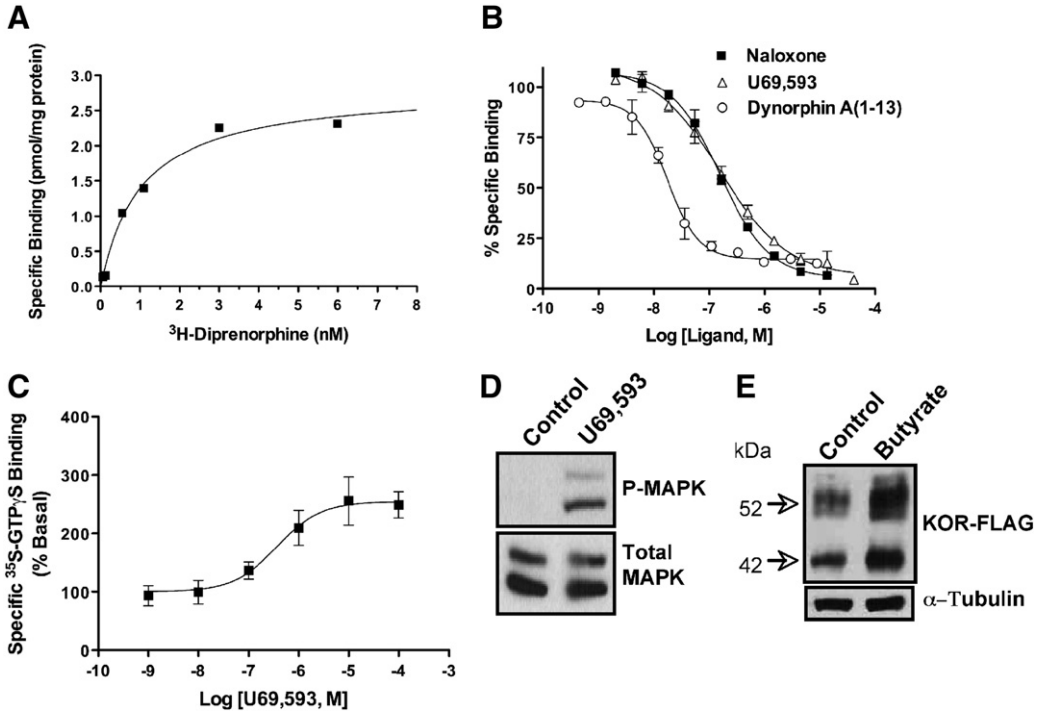

Fig. 1 – Characterization of the epitope-tagged rKOR. (A) Membranes were prepared from HEK 293 cells expressing rKOR and assayed by saturation analysis using [^{3}H]diprenorphine (0.05–7 nM). Data are representative of ten independent experiments. (B) rKOR membrane fractions were used to measure the apparent dissociation constant (K_i) of dynorphin A (1–13), U69,593 and naloxone. Ten concentrations (between 0.5 nM and 40 μM) of each ligand were assayed for displacement of [^{3}H]diprenorphine binding (7 nM). IC_{50} values were determined by non-linear regression analysis of the displacement curves using Prism 3.0 and K_i values were calculated using the Cheng–Prusoff equation ($n=3$). (C) Membrane fractions were incubated with 0.3 nM [^{35}S] GTPγS and 10 μM GDP in the absence or presence of varying concentrations of U69,593 at 30 °C for 90 min. Reactions were terminated by filtration and filter-bound radioactivity was determined by liquid scintillation counting. Dose–response curves were analyzed by non-linear regression using Prism 3.0 (GraphPad Software) ($n=3$). (D) rKOR cells in serum-free media were treated with or without 1 μM U69,593 for 10 min followed by extraction with 1% DDM lysis buffer. Whole cell lysates were prepared and subjected to SDS/PAGE and western blot analysis using antibodies that recognize phosphorylated MAPK or total (phosphorylated and nonphosphorylated) MAPK. Data are representative of three independent experiments. (E) Cells were incubated with or without 1 mM sodium butyrate for 18 h. Whole cell lysates were subjected to SDS/PAGE and western blot analysis using the anti-FLAG M1 antibody to detect rKOR, or α-tubulin antibody to control for the amount of protein loaded in each lane. Data are representative of three independent experiments.

receptor kinases. Receptor phosphorylation leads to the recruitment of arrestin, which induces uncoupling of the receptor from the G-protein. This phenomenon is known as desensitization and has been shown to contribute to the development of analgesic tolerance in mice (McLaughlin et al., 2004). Desensitization has also been clearly shown in cell culture systems expressing the human KOR (reviewed in Liu-Chen, 2004), however, studies involving the rat kappa opioid receptor have been less consistent, with some studies demonstrating agonist-induced desensitization (Appleyard et al., 1999; McLaughlin et al., 2003) and others not (Li et al., 2002). Prolonged agonist treatment of cells expressing the rat kappa receptor led to the recruitment of arrestin to the phosphorylated receptor which also initiated receptor endocytosis via clathrin-coated pits in a dynamin-dependent manner (McLaughlin et al., 2003). Once inside the cell, opioid receptors are either degraded or recycled back to the plasma membrane (reviewed by Williams et al., 2001).

Surprisingly, chronic agonist treatment leads to receptor down-regulation in the case of the human, but not the mouse or rat KOR (Zhang et al., 2002; Chen et al., 2006; Wannemacher et al., 2007). The carboxyl terminus of the receptor is believed to contribute towards the species variation (Zhang et al., 2002). The mechanism for the receptor proteolysis associated with down-regulation has been generally assumed to involve internalization of receptors into endosomes, fusion of endosomes with lysosomes and degradation of the receptor via lysosomal proteases. However, our laboratory has reported evidence implicating the ubiquitin/proteasome system in agonist-induced μ- and δ-opioid receptor down-regulation (Chaturvedi et al., 2001b). We have further shown that co-treatment of HEK 293 cells stably expressing δ-opioid receptor with ZLLL, a proteasome inhibitor, prevents the development of tolerance following chronic treatment with DADL (Yadav et al., 2007). Down-regulation of the human κ-opioid receptor has also been shown to be partially attenuated by co-treatment with a proteasome inhibitor (Li et al., 2000), and polyubiquitination of the receptor is associated with down-regulation (Li et al., 2008). Additionally, we have provided evidence that the proteasome is also involved in basal turnover of the rat κ-opioid receptor (Wannemacher et al., 2007).

Mass spectrometry (MS) has become a powerful tool for the characterization of proteins. Digestion of a purified protein with proteases that cleave at specific amino acid residues yields peptides that can be identified and sequenced using tandem MS/MS analysis. GPCRs are notoriously difficult to purify and to characterize using physical methods, however, some progress has been made. MS analysis has been reported for rhodopsin (Ablonczy et al., 2005), the β-2 adrenoceptor (Trester-Zedlitz et al., 2005), the cannabinoid CB2 receptor (Zvonok et al., 2007), the μ-opioid receptor (Christoffers et al., 2003) and the δ-opioid receptor (Christoffers et al., 2005). To date, rhodopsin (Palczewski et al., 2000) and the β2-adrenergic receptor (Rasmussen et al., 2007; Cherezov et al., 2007) remain the only GPCRs whose structures have been solved by X-ray crystallography. In addition to the structural instability inherent in GPCRs, obtaining sufficient amounts of purified receptor for crystallographic studies has been problematic.

In this study, we report the purification of rKOR using sequential wheat germ agglutinin (WGA) chromatography, Sephacryl S300 gel filtration chromatography, anti-FLAG immunoaffinity chromatography and SDS/PAGE. Furthermore, we have identified and characterized peptides derived from the purified receptor using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry and confirmed their sequence using tandem mass spectrometry. In addition, we have identified HSP70 in the purified KOR preparation and in rKOR immunoprecipitates, suggesting that HSP70 forms protein–protein interactions with the receptor.

2. Results

2.1. *Characterization of rKOR in stably transfected HEK 293 cells*

A clonal HEK 293 cell line was established that stably expressed rKOR with a FLAG tag at the amino terminus. [^{3}H] Diprenorphine bound to the epitope-tagged receptor with an apparent dissociation constant (K_d) of 1.1±0.2 nM, and the maximum number of binding sites (B_{max}) was 2.6±0.4 pmol/mg protein (Fig. 1A). To test the affinity of the FLAG-tagged rKOR for known KOR ligands, competition analysis was performed using dynorphin A (1–13), U69,593 and the non-selective opioid antagonist, naloxone. These compounds inhibited diprenorphine binding to rKOR with K_i's of 2.0±0.4, 18±3 and 18±2 nM, respectively (Fig. 1B). To confirm that the epitope-tagged rKOR was capable of downstream signaling following agonist activation, G-protein coupling and activation of the MAP kinase pathway was examined. The non-peptide arylacetamide KOR agonist, U69,593, increased maximal [^{35}S]GTPγS binding 2.5-fold with an EC_{50} of 330 nM (Fig. 1C). In addition, stimulation of rKOR cells for 10 min with

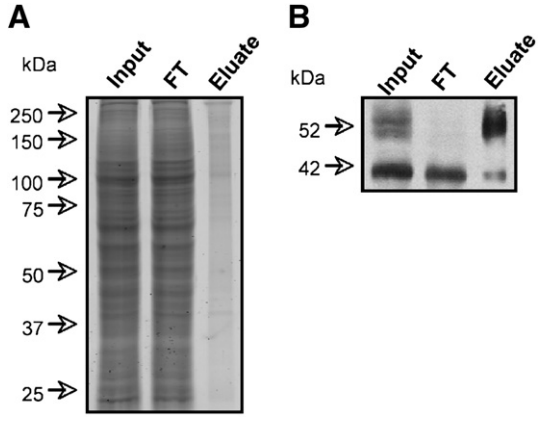

Fig. 2 – Purification of rKOR using WGA chromatography. rKOR membranes were prepared and extracted with 1% DDM. Following ultracentrifugation, soluble membrane protein (300 mg) was incubated with WGA-agarose beads. The beads were washed extensively, then eluted with buffer containing 0.5 M *N*-acetylglucosamine. (A) WGA input, flow-through and eluate fractions (0.03% v/v) were analyzed by 12% SDS/PAGE and stained with Sypro Ruby. (B) Aliquots (0.03% v/v) of each WGA fraction were analyzed by western blot analysis using anti-FLAG M1 antibody for detection.

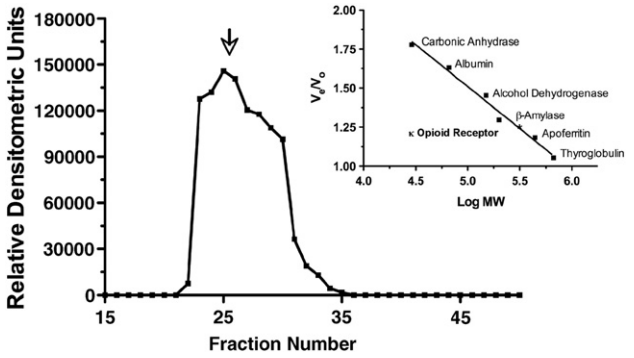

Fig. 3 – Gel filtration chromatography of rKOR. The WGA eluate was applied to a Sephacryl S300 column and fractions were assayed by western blot analysis using anti-FLAG M1 antibody. *Inset:* The Sephacryl S300 column was calibrated using carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, beta-amylase, apoferritin and thyroglobulin as protein standards. Using this standard curve (r^2=0.98), the calculated molecular weight of rKOR was 360 kDa. The arrow denotes the elution volume from which the molecular weight of rKOR was calculated.

U69,593 (1 μM) caused a 10-fold increase in the level of phosphorylated MAP kinase as indicated by western blotting using an antibody that specifically recognized the phosphorylated, active form of MAP kinase (Fig. 1D, upper panel). The use of an antibody that recognized all forms of MAP kinase revealed that there was no change in the level of total MAP kinase immunoreactivity (Fig. 1D, lower panel).

To facilitate the purification scheme, it was desirable to maximize the level of rKOR protein expression. To augment receptor expression, we took advantage of the observation that histone deacetylase (HDAC) inhibitors have been shown to increase the expression of many genes, including those that are under the control of a cytomegalovirus promoter (Choi et al., 2005). As shown in Fig. 1E, treatment of rKOR cells for 18 h with 1 mM sodium butyrate, an HDAC inhibitor, increased the level of the 42 and 52 kDa rKOR species approximately 2-fold, while levels of α-tubulin remained unchanged.

2.2. *Receptor purification*

The nonionic detergent, *n*-dodecyl-β-D-maltopyranoside (DDM), was selected for solubilization of rKOR based on its ability to effectively solubilize the receptor and its compatibility with subsequent mass spectrometric analysis. Membranes from rKOR cells were prepared, solubilized with a lysis buffer containing 1% DDM and subjected to ultracentrifugation. The ultracentrifugal supernatant, containing approximately 300 mg of solubilized membrane protein, was incubated with WGA-agarose beads to enrich the 52 kDa mature form of rKOR. We have shown previously that the 42 kDa does not bind efficiently to WGA (Wannemacher et al., 2007). Aliquots (0.03% v/v) of the WGA-agarose input, flow-through fraction and *N*-acetylglucosamine eluate were resolved using SDS/PAGE to assess the effectiveness of the lectin chromatography to purify the receptor. Sypro Ruby staining of the SDS/PAGE gel revealed that greater than 90% of the protein loaded on the column was present in the flow-through fraction (Fig. 2A). Western blot analysis indicated that the 42 kDa species was present nearly quantitatively in the flow-through fraction while the 52 kDa species was undetectable, indicating that the 52 kDa species avidly bound to the WGA-agarose. The 52 kDa receptor was eluted from the resin with 0.5 M *N*-acetylglucosamine, and recovery was approximately 95%, based on the yield of immunoreactivity. Quantification of the amount of protein loaded per lane of the gel revealed that the input and flow-through fractions contained 80 μg while the eluate contained 4 μg of protein. Given that the aliquot of the eluate analyzed contained 1/20th of the amount of input protein but yielded equivalent amounts of rKOR immunoreactivity, the purification factor for the WGA chromatography was estimated to be approximately 20-fold.

The next step in the purification procedure was gel filtration chromatography. Sephacryl S300 resin was employed since it provided sufficient separation of rKOR from proteins in the void volume. Calibration of the Sephacryl S300 column with protein standards (r^2=0.98) indicated that the rKOR enriched by lectin chromatography migrated with an apparent molecular mass of approximately 360 kDa (Fig. 3, inset). Aliquots of each gel filtration fraction were resolved by

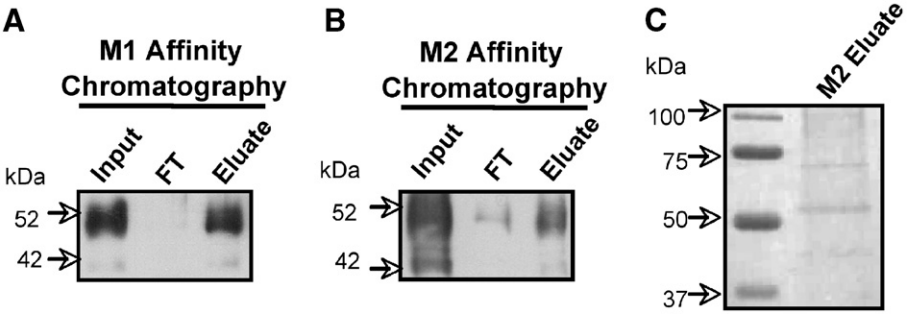

Fig. 4 – Anti-FLAG immunoaffinity chromatography of rKOR. Pooled gel filtration fractions were incubated with anti-FLAG M1 agarose. The beads were washed thrice with column wash buffer and then eluted with buffer containing 20 mM EDTA. (A) Aliquots (0.03% v/v) of the input, flow-through and eluate fractions were analyzed by western blotting using the anti-FLAG M1 antibody. The eluate was concentrated using a 9000 MW cutoff column and then incubated with anti-FLAG M2 agarose. Beads were washed extensively, then rKOR was eluted with SDS/PAGE loading buffer. (B) Aliquots (1.5% v/v) of the input, flow-through and eluate were analyzed by western blotting using the anti-FLAG M1 antibody. (C) The anti-FLAG M2 eluate was resolved by 12% SDS/PAGE and stained with E-Zinc protein stain.

SDS/PAGE and analyzed using western blotting to detect rKOR (Fig. 3). Fractions 23–30 contained the majority of rKOR immunoreactivity and were pooled for further purification steps. Recovery of rKOR after gel filtration was 70% and the receptor was enriched approximately 3-fold.

The pooled gel filtration fractions were subjected to anti-FLAG M1 immunoaffinity chromatography. Since binding to the M1 antibody is Ca^{2+} dependent, the bound receptor was eluted with 20 mM EDTA to chelate the free Ca^{2+}. It should be noted that CHAPS was used in place of DDM during the M1 wash and elution steps as a small detergent micelle size was required for subsequent purifications steps. Aliquots (0.03% v/v) of the input, flow-through fraction and eluate were resolved using SDS/PAGE and the receptor was visualized by western blotting (Fig. 4A).

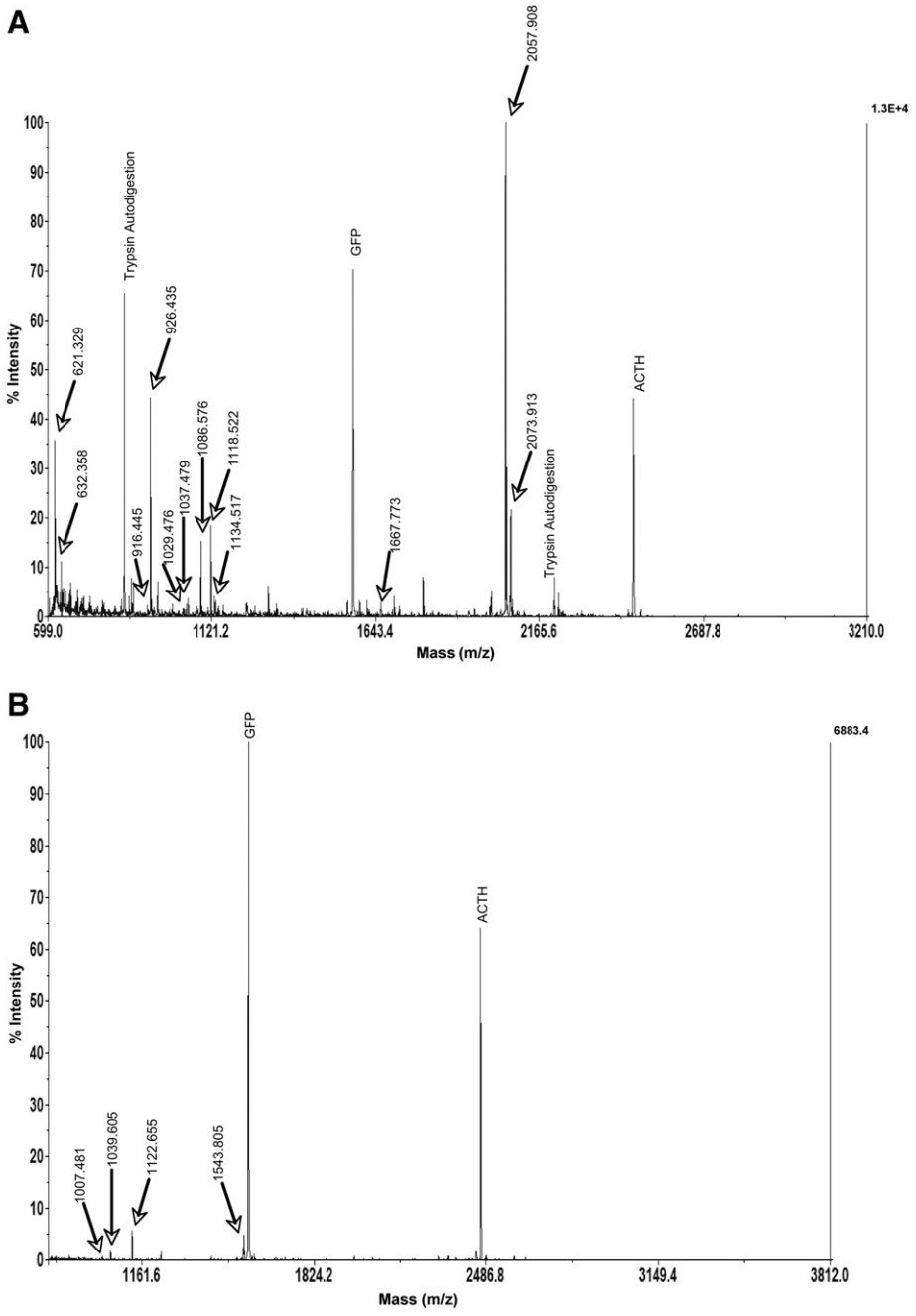

Fig. 5 – Mass spectra from trypsin or sequential trypsin and cyanogen bromide digests. The 52 kDa protein was excised from the gel and digested with trypsin for 16 h. The resulting peptides were desalted, mixed with MALDI matrix and mass spectra were acquired on an Applied Biosystems 4800 Proteomics Analyzer with TOF/TOF optics. The remaining gel fragments were further digested with cyanogen bromide for 24 h. The resulting peptides were analyzed as described above. (A) Representative MS spectrum following trypsin digestion and C_4 desalting. Peptide peaks derived from rKOR are labeled, as well as peaks originating from trypsin autodigestion and internal controls. (B) Representative MS spectrum following sequential trypsin and cyanogen bromide digestion is displayed with internal standards and rKOR peaks labeled.

Table 1 – rKOR peptides identified by mass spectrometry

Mass	Amino acid (AA) sequence	AA number, location
621.329	ALDFR	174–178, ICL2
632.358	LLSGSR	266–271, ICL3
916.445	DVGGMNKPV	380–388, C-terminus
926.435	DFCFPIK	351–357, C-terminus
932.451	DVGGMNKPV (MSO)	380–388, C-terminus
1007.481	PFQSAVYLM*	121–129, TM2
1029.476	YIAVCHPVK	165–173, ICL2
1037.479	MERQSTNR (MSO)	360–367, C-terminus
1039.605	FVIIRYTK*	90–97, TM1/ICL1
1086.576	YIAVCHPVK (CAM)	165–173, ICL2
1118.522	NTVQDPASMR	370–379, C-terminus
1122.655	FVIIRYTKM*	90–98, TM1/ICL1
1134.517	NTVQDPASMR (MSO)	370–379, C-terminus
1373.665	VRNTVQDPASMR	368–379, C-terminus
1543.805	SVDRYIAVCHPVK* (CAM)	161–173, TM3/ICL2
1667.773	DDDDAMESPIQIFR (MSO)	4–17, N-terminus
2057.908	DYKDDDDAMESPIQIFR	1–17, N-terminus
2073.913	DYKDDDDAMESPIQIFR (MSO)	1–17, N-terminus

Peptides matching within 30 ppm of the theoretical digest of rKOR are shown. Peptides denoted with an asterisk were detected following sequential digestion with trypsin and cyanogen bromide. All other peptides originated from a digest with trypsin alone. Note that the numbering system accounts for the presence of the FLAG epitope at the amino terminus and thus will differ from the numbering of the wild type receptor by 8 amino acids. TM, transmembrane domain; ICL, intracellular loop; MSO, methionine sulfoxide; CAM, carbamidomethyl.

Recovery of the receptor in the eluate was estimated to be 80%, based on the yield of immunoreactivity. Assessment of the purification factor at this stage was difficult due to the low levels of protein in the eluate, however, when anti-FLAG M1 affinity chromatography was used at an early stage of purification, the receptor was enriched approximately 50-fold.

At this point, the receptor was highly enriched, however, the volume needed to be reduced in order to perform preparative SDS/PAGE. Therefore, the M1 eluate was loaded onto a 9000 MW cutoff column. However, as the volume was reduced, it was evident that there was significant concentration of the CHAPS detergent, which would likely cause problems during the subsequent SDS/PAGE separation. As a result, anti-FLAG M2 immunoaffinity chromatography was used to reduce the concentration of CHAPS (Fig. 4B). The bound receptor was eluted with 1 mM FLAG peptide in 60 μl of SDS/PAGE loading buffer with a recovery of approximately 40%. The eluate was resolved by SDS/PAGE and stained with E-Zinc protein stain (Pierce Biotechnology, Rockford, IL). Protein bands migrating at approximately 52 and 70 kDa were clearly evident, along with other minor bands (Fig. 4C).

2.3. *Enzymatic digestion, chemical cleavage and mass spectrometry of the purified receptor*

The 52 kDa band was excised from the gel and digested with trypsin. Following digestion and extraction of the resulting peptides, the gel fragments were further subjected to chemical cleavage using cyanogen bromide. The extracted peptides from each digest were desalted and then analyzed on an Applied Biosystems 4800 Proteomics Analyzer with TOF/TOF optics. A theoretical digest of rKOR using the FindMod tool available from the ExPASy Proteomics Server (http://www.expasy.org/), was performed and compared with the spectra obtained. A total of 18 peptides from the trypsin digestion were detected that matched within 30 parts-per-million (0.03 Da at 1000 *m/z*) of the theoretical digest (Fig. 5A and Table 1). Four additional peptides derived from rKOR were detected in the cyanogen bromide digest (Fig. 5B and Table 1). These peptides were the result of receptor cleavage with either cyanogen bromide alone or in combination with trypsin.

For further confirmation of the identity of rKOR peptide fragments, peptides were analyzed by precursor-selective tandem mass spectrometry. The MS/MS spectra obtained from the 2057.91 Da peptide, DYKDDDDAMESPIQIFR, derived from the N-terminus of FLAG-tagged rKOR is shown in Fig. 6. Extensive y-ion series coverage confirmed the sequence of this peptide.

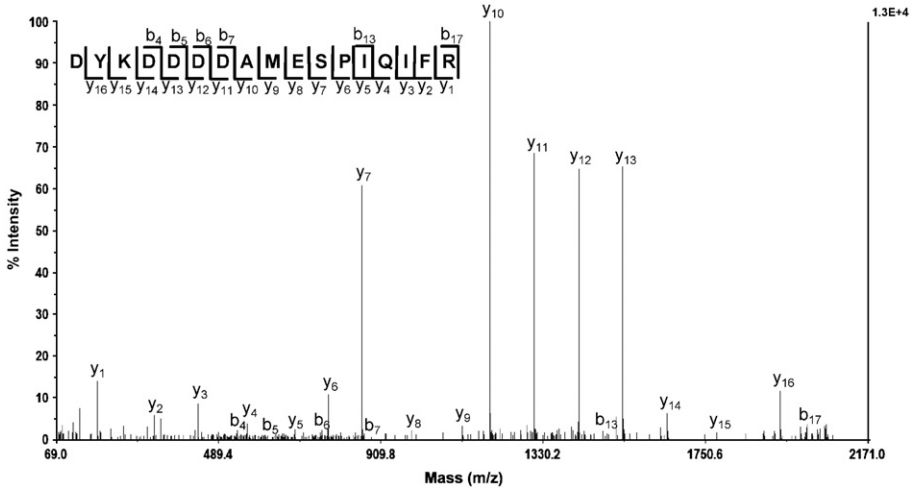

Fig. 6 – MS/MS spectrum of a tryptic peptide derived from rKOR. A tryptic peptide of 2057.91 Da derived from the N-terminus of rKOR was selected for tandem MS/MS analysis. The fragmentation pattern confirmed the amino acid sequence of the peptide. *Inset:* Amino acid sequence of the parent peptide showing b- and y-ion series coverage.

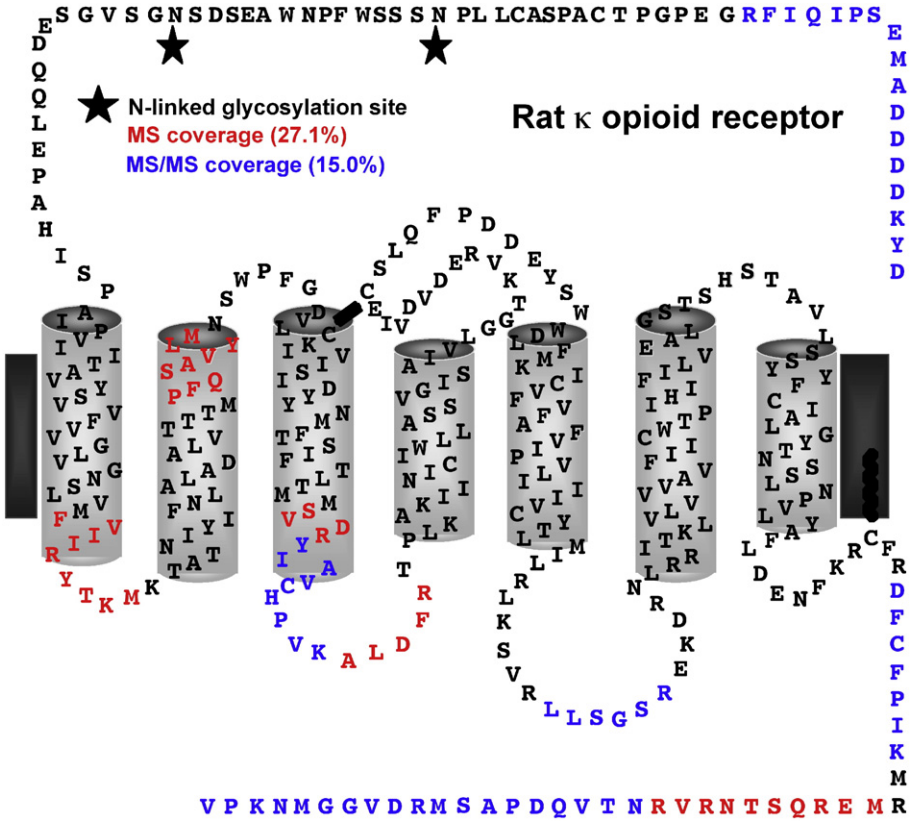

Fig. 7 – Schematic of rKOR amino acid sequence showing coverage by mass spectrometry. Peptide sequences detected by MS analysis and confirmed by MS/MS are shown in blue and account for 15.0% of rKOR. Peptide sequences detected by MS analysis alone are shown in red and together with the peptides confirmed by MS/MS, account for 27.1% of rKOR.

As depicted in Table 1 and Fig. 7, the sequential use of trypsin and cyanogen bromide allowed for the detection of several transmembrane peptides. The lack of amino-terminal coverage was not unexpected given the relative lack of lysine, arginine and methionine residues, coupled with the presence of N-linked glycosylation which obviously alters the mass of peptides derived from the receptor amino terminus in an unpredictable manner. Despite the difficulty in mass spectrometric analysis of membrane proteins, peptides obtained from the digestion of rKOR with trypsin and cyanogen bromide combined to constitute 27.1% of the rKOR amino acid sequence (Fig. 7).

2.4. *Identification of HSP70 as a KOR-interacting protein*

In addition to KOR, a protein band migrating at approximately 70 kDa was seen in the M2 eluate (Fig. 4C). Trypsin digestion and mass spectrometric analysis identified this protein as HSP70. A total of 41 peptides from the trypsin digestion were detected that matched within 30 parts-per-million of the HSP70 theoretical digest, accounting for 61.3% sequence coverage of HSP70 by MS analysis. To verify the identification, ten MS/MS spectra were acquired and all were confirmed to correspond to HSP70 peptides. The MS/MS spectrum of the HSP70 peptide, NALESYAFNMK (1287.61 Da), is shown in Fig. 8A.

Following the identification of HSP70 in the highly enriched receptor preparation by mass spectrometry, we next sought to detect HSP70 following anti-FLAG M1 immunoprecipitation of rKOR using an anti-HSP70 antibody. As displayed in Fig. 8B, HSP70 was detected in M1 eluates from rKOR cells, but was notably absent in M1 immunoprecipitates derived from nontransfected HEK 293 cells.

3. Discussion

In this study, a clonal HEK 293 cell line was established that expressed rKOR containing a FLAG epitope at the amino terminus to facilitate western blot analysis and immunoaffinity chromatography. The level of expression was relatively high and the apparent dissociation constant (K_d) for [^{3}H] diprenorphine binding was in reasonable agreement with previously published values (Raynor et al., 1994). The receptor also displayed high affinity binding of U69, 593, dynorphin A (1–13) and naloxone, again consistent with affinities reported previously (Raynor et al., 1994). In addition, G-protein coupling and MAP kinase assays indicated that the tagged receptor was fully functional and capable of agonist-induced downstream signal transduction.

In the course of these studies, we observed that overnight treatment of rKOR cells with the HDAC inhibitor, sodium

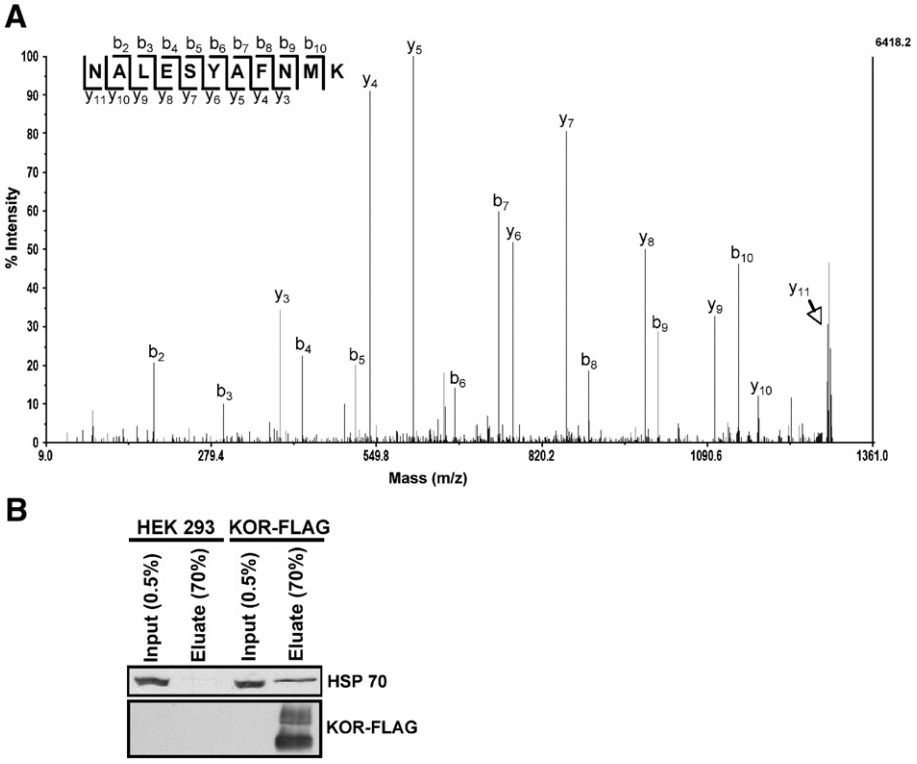

Fig. 8 – Evidence for interaction of rKOR with HSP70. (A) MS/MS spectrum of a tryptic peptide derived from HSP70 that co-purified with rKOR. A tryptic peptide of 1287.61 Da derived from the HSP70 was selected for tandem MS/MS analysis. The fragmentation pattern confirmed the amino acid sequence of the peptide. *Inset:* Amino acid sequence of the parent peptide showing b- and y-ion series coverage. (B) HSP70 co-immunoprecipitated with rKOR. Cells lysates were prepared from HEK 293 cells and rKOR cells by extraction with RIPA buffer and lysates were incubated with anti-FLAG M1 agarose. Beads were washed extensively, then rKOR was eluted with SDS/PAGE loading buffer. Aliquots of the input (0.5% v/v) and eluate (70%) were analyzed by western blotting using anti-HSP70 and anti-FLAG M1 antibody.

butyrate, increased the apparent receptor B_{max} by approximately 2-fold, therefore, we exploited this up-regulation of receptor expression in our purification scheme. We have also observed that other HDAC inhibitors, such as trichostatin and sodium valproate, induced similar increases in receptor expression, and that the increase in protein expression was accompanied by a similar increase in rKOR mRNA levels (data not shown).

rKOR was extracted from transfected HEK cell membranes with *n*-dodecyl-β-D-maltopyranoside. Although the solubilized receptor initially retained the capacity for high affinity [^{3}H]diprenorphine binding, the ligand binding activity was labile, presumably as a result of a time-dependent denaturation of the ligand binding pocket. Further studies will be necessary to uncover methods to stabilize the receptor's ligand binding activity subsequent to membrane extraction.

Given that the B_{max} of the rKOR cells used in these studies was 2.6 pmol/mg protein, the 52 kDa receptor would theoretically need to be purified approximately 7,400-fold to achieve homogeneity. This estimation assumes that all receptors present in the cell are capable of high affinity ligand binding. Due to the labile nature of the ligand binding pocket following solubilization, rKOR was detected by immunoblot analysis during each stage of purification. Based on immunoreactivity, we calculated that rKOR was purified 20-fold, 3-fold and 50-fold by WGA chromatography, gel filtration chromatography and anti-FLAG M1 immunoaffinity chromatography, respectively. As a result of these first steps of the purification, we calculate that rKOR was purified approximately 3000-fold prior to anti-FLAG M2 immunoaffinity chromatography and SDS/PAGE.

Wheat germ agglutinin chromatography was the first step in the purification of rKOR. Solubilized opioid receptors have been demonstrated previously to bind to WGA (Gioannini et al., 1982). We have shown previously that the mature forms of the mu and delta opioid receptors also bound tightly to and could be enriched following elution from WGA-agarose columns (Christoffers et al., 2003; Christoffers et al., 2005). This provides further confirmation that the mature rKOR expressed on the cell surface is an N-linked glycoprotein (Wannemacher et al., 2007).

Sephacryl S300 gel filtration chromatography indicated that rKOR migrated with an apparent molecular mass of 360 kDa, which was in good agreement with a previous estimate of 400 kDa for the kappa receptor extracted from guinea pig brain (Itzhak et al., 1984). In this study we found that the apparent molecular mass of rKOR was 42 and 52 kDa, based on SDS/PAGE migration. Simon et al. (1990) reported that the kappa receptor purified from frog brain appeared on SDS/PAGE to have a mass of 65 kDa. The theoretical molecular

weight of FLAG-tagged rKOR without any post-translational modifications is 43,612 Da. Since the concentration of DDM in the extraction buffer was considerably greater than its critical micelle concentration (CMC), the solubilized receptor was contained presumably within DDM micelles, which have a molecular mass of 40–76 kDa (CMC=0.006%, FW=510.6, aggregation number=78–149). Even when taking into account the size of the DDM micelle, it was apparent that the receptor was either forming multimers with itself or was associating with other proteins in the extract. Gel filtration fractions containing rKOR were pooled and further purified using anti-FLAG immunoaffinity chromatography and preparative SDS/PAGE. A similar purification scheme has been employed previously by our laboratory to purify murine μ- and δ-opioid receptors (Christoffers et al., 2003; Christoffers et al., 2005).

In this study, sequential enzymatic and chemical cleavage, using trypsin and cyanogen bromide, respectively, was used to digest the gel-purified rKOR. Digestion with trypsin resulted in extensive sequence coverage of the intracellular loops and the carboxyl terminus of the receptor. Enzymatic digestion of GPCRs with trypsin, however, is problematic due to the lack of lysines and arginines within transmembrane domains. As a result, trypsin digestion can generate large hydrophobic peptides that do not ionize well using MALDI mass spectrometry. An alternative to enzymatic digestion is chemical digestion. Cyanogen bromide, which cleaves at methionine residues, has been used to obtain extensive sequence coverage of rhodopsin, a GPCR (Kraft et al., 2001). However, not all GPCRs have an adequate number of methionines sufficiently spaced throughout the amino acid sequence to allow for cyanogen bromide cleavage that will yield appropriately sized peptides for MS analysis. Substantial sequence coverage of integral membrane proteins has been reported using a combination of trypsin and cyanogen bromide cleavage (van Montfort et al., 2002; Quach et al., 2003), as was the case in our present study.

Mass spectrometry has become an invaluable tool to examine post-translational modifications (PTMs) of proteins (Jensen, 2004). The software tool, FindMod, takes into account mass shifts due to PTMs when matching acquired data to the theoretical digest (Wilkins et al., 1999). As receptor phosphorylation has been suggested to play an important role in the development of opioid tolerance (reviewed in Wang and Wang, 2006), the identification of phosphorylation sites within rKOR was of particular interest. The addition of the mass of a phosphate group (79.97 Da) to a given peptide containing Ser, Thr or Tyr is suggestive of protein phosphorylation. In this study, however, we were unable to detect evidence for phosphorylation of the receptor under basal conditions. We did, however, obtain substantial sequence coverage of the receptor carboxyl-terminal domain and intracellular loops, two likely regions of agonist-induced phosphorylation. Therefore, similar techniques will be employed to examine sites of receptor phosphorylation under agonist-stimulated conditions. Ser 369 in the kappa opioid receptor has been shown to be phosphorylated following agonist treatment (Appleyard et al., 1999). In addition, tyrosine phosphorylation of rKOR at Tyr 87 and Tyr 157 has been suggested to be involved in insulin potentiation of rKOR-activated G-protein-gated inwardly rectifying potassium channels (Appleyard et al., 2000).

Our laboratory has reported that μ- and δ-opioid receptors were degraded by the proteasome under basal conditions and following agonist stimulation (Chaturvedi et al., 2001b), and we have shown recently that rKOR was also degraded by the proteasome under basal conditions (Wannemacher et al., 2007). Proteins are targeted for degradation by the proteasome through the covalent attachment of polyubiquitin chains, which occurs predominately on lysine residues. The carboxyl-terminal glycine of ubiquitin serves as the site of attachment of the first ubiquitin moiety to target proteins and is preceded by a glycine and an arginine residue. During trypsin digestion, ubiquitin should be cleaved at the arginine residue leaving a glycine dipeptide attached to the target protein. This addition of 114.04 Da, as well as a trypsin mis-cleavage in the target protein due to the loss of a positive charge on the lysine, is characteristic of ubiquitination (Peng et al., 2003). Furthermore, additional trypsin mis-cleavage at the distal arginine in ubiquitin can result in the addition of LRGG to the target protein, thus resulting in a mass increase of 383.4 Da (Jeon et al., 2007). It has been reported recently that agonist-induced ubiquitination of the human KOR was reduced, but not totally abolished, when three lysine residues in the carboxyl-terminal domain were mutated to arginine (Li et al., 2008). In this study, we were unable to find evidence for ubiquitination of the rKOR under basal conditions. It is also possible that proteasomal degradation of rKOR is mediated by the polyubiquitination of a protein that interacts strongly with rKOR. Mutation of all cytoplasmic lysines (Tanowitz and Von Zastrow, 2002) suggested that the delta opioid receptor was not directly ubiquitinated, despite our observation that it is degraded by the proteasome, both under basal and agonist-stimulated conditions (Chaturvedi et al., 2001b).

During the course of the KOR purification, we identified HSP70 as a KOR-interacting protein by mass spectrometry and western blot analysis using an anti-HSP70 antibody. HSP70 is expressed in all cells, both eukaryotic and prokaryotic, and is involved in protein folding and translocation. While HSP70 is expressed in the absence of heat shock, increased levels can be found following a variety of pathological and environmental insults (reviewed in Kiang and Tsokos, 1998). HSP70 has been shown to interact with the angiotensin II type 1 receptor (Lanctot et al., 2006) as well as rhodopsin (Chapple and Cheetham, 2003), both GPCRs. The μ opioid receptor has also been reported to interact with hlj1, a member of the closely related HSP40 family (Ancevska-Taneva et al., 2006). HSP70 has also been linked to the ubiquitin/proteasome pathway via its interactions with BAG-1 and the ubiquitin ligase CHIP (Demand et al., 2001). The connection of HSP70 with proteasome-mediated degradation is of particular interest as we have shown previously that rKOR is degraded by the proteasome (Wannemacher et al., 2007). It should be noted that although HSP70 shares a high degree of sequence similarity with heat shock cognate 70 (HSC70), mass spectrometric analysis conclusively showed that KOR interacted with HSP70, since 34 of the 41 HSP70 peptides identified by MS analysis are derived exclusively from HSP70.

In summary, we have purified rKOR from stably transfected HEK 293 cells and have characterized peptides resulting from enzymatic and chemical cleavage by mass spectrometry.

Additional studies will be necessary to identify and confirm sites of agonist-induced post-translational modifications.

4. Experimental procedures

4.1. Materials

Dynorphin A (1–13) was a product of Multiple Peptide Systems (San Diego, CA). Naloxone and U69,593 [(5α,7α,8β)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide] were obtained from the National Institute on Drug Abuse (Bethesda, MD). *n*-Dodecyl-β-D-maltopyranoside was purchased from Anatrace (Maumee, OH).

4.2. Cell culture and transfection

HEK 293 cells were transfected with an expression plasmid encoding the rat kappa opioid receptor tagged with an N-terminal FLAG epitope (kindly provided by Dr. Lakshmi Devi, Mt. Sinai Medical Center, NY) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). A clonal cell line stably expressing the FLAG-tagged rat kappa opioid receptor (rKOR cells) was obtained following selection in media containing 1 mg/ml G418 (Invitrogen). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 0.25 mg/ml G418. For receptor purification, cells were placed in serum-free media containing 1 mM sodium butyrate 18 h prior to harvesting and preparation of membranes.

4.3. Membrane preparation and radioligand binding assays

rKOR cells were harvested in phosphate-buffered saline (PBS) and centrifuged at 500 ×*g* for 5 min. Cell pellets were resuspended in PBS and centrifuged as above. The cell pellets were homogenized with a Tekmar tissuemizer (Cincinnati, OH) in chilled 50 mM Tris–HCl, pH 7.5, and a membrane fraction was prepared by ultracentrifugation of the homogenate at 100,000 ×*g* for 30 min at 4 °C. The membrane pellet was washed with chilled 50 mM Tris–HCl, pH 7.5, and resuspended by homogenization in ice-cold 0.32 mM sucrose, 50 mM Tris–HCl, pH 7.5. Membrane preparations were stored at −80 °C if not used immediately. The protein concentration of the membrane preparations was determined using the D_c protein assay (BioRad, Hercules, CA) with bovine serum albumin as the standard.

Radioligand binding assays were conducted in a final volume of 0.25 ml, using rKOR cell membrane preparations diluted with 50 mM Tris–HCl, pH 7.5, to contain 60–80 μg protein/ml. Saturation binding assays were conducted in duplicate at room temperature using concentrations of [15,16-^{3}H]-diprenorphine (specific activity 50.0 Ci/mmol, Perkin Elmer, Boston, MA) ranging from 0.05 nM to 7 nM. Samples containing tritiated diprenorphine in the presence of excess unlabeled cyclazocine (1 μM) were assayed to determine non-specific binding, which was subtracted from total binding to obtain specific binding. Following incubation for 30 min to reach equilibrium, binding assays were terminated by filtration through Whatman GF/B filters (VWR International, Buffalo Grove, IL). Filters were immersed in Ecoscint H liquid scintillation cocktail (National Diagnostics, Somerville, NJ) prior to determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. Saturation curves were analyzed by non-linear regression using Prism 3.0 (GraphPad Software, San Diego, CA) to determine B_{max} and K_d values.

For competition analysis, rKOR cell membranes were prepared as described above. Ten concentrations (between 0.5 nM and 40 μM) of each ligand were assayed for displacement of [^{3}H]diprenorphine (7 nM). Binding assays were conducted as described above. IC_{50} values were determined by non-linear regression analysis of the displacement curves using Prism 3.0, and K_i values were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

4.4. Agonist-induced stimulation of [^{35}S]GTPγS binding

rKOR cell membrane fractions were prepared and [^{35}S]GTPγS binding assays were conducted as described previously (Yadav et al., 2007). Briefly, rKOR membrane fractions (7.5 μg protein) were incubated with 0.3 nM [^{35}S]GTPγS (specific activity 1117 Ci/mmol, Amersham Bioscience, Piscataway, NJ) and 10 μM GDP (Calbiochem, La Jolla, CA) in the absence or presence of varying concentrations of U69,593 (ranging from 1 nM to 100 μM) in 1 ml of 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.1% BSA, 1 mM DTT and 0.025% digitonin. Reactions were incubated at 30 °C for 90 min. Nonspecific binding was determined by incubation of samples in the presence of 15 μM unlabeled GTPγS, and was subtracted from total basal and total agonist-stimulated binding. Reactions were terminated by filtration through Whatman GF/B filters. Filters were immersed in Ecoscint H liquid scintillation cocktail prior to determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. Dose–response curves were analyzed by non-linear regression using Prism 3.0 (GraphPad Software) to determine E_{max} and EC_{50} values.

4.5. MAP kinase assays

rKOR cells were placed in serum-free media overnight to reduce basal MAP kinase signaling. The next day, the media was replaced with fresh serum-containing media with or without 1 μM U69,593 and cells were incubated at 37 °C for 10 min. Reactions were terminated by aspiration of the media and solubilization of the cells on the dish with 1% *n*-dodecyl-β-D-maltopyranoside (DDM), 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail (Sigma, 1:100) and phosphatase inhibitor cocktail 1 and 2 (Sigma, each 1:100). The detergent lysate was centrifuged at 16,000 ×*g* for 20 min, and the supernatant was recovered. The protein concentration in the supernatant was determined using the D_c protein assay (BioRad). SDS/PAGE and western blotting were conducted as described previously (Yadav et al., 2007) using 60 μg of protein per lane. Mouse monoclonal anti-phospho-MAP kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-MAP kinase antibody that recognizes total (phosphorylated and nonphosphorylated) MAP kinase (Upstate Biotechnology, Charlottesville, VA) were

used as primary antibodies. Goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) or goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) were used as secondary antibodies.

4.6. Western blotting

For detection of rKOR by western blotting, samples were heated at 50 °C for 5 min in gel loading buffer, resolved by 12% SDS/PAGE and transferred to Immobilon P polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked for 30 min in 3% dried milk, 10% glycerol, 0.5% Tween-20, 1 M glucose, 50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$ and 0.005% thimersol prior to overnight incubation with anti-FLAG M1 antibody (Sigma). Membranes were washed in TBST then incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) for 1 h at room temperature. Membranes were washed and developed using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) and HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ). Blots were quantitated using SynGene Software (Synoptics Ltd., Cambridge, England).

4.7. Membrane preparation and wheat germ agglutinin (WGA)-agarose chromatography

Cells were harvested in 50 mM Tris–HCl, pH 7.5 and membranes were prepared by homogenization with a Tekmar tissuemizer (Cincinnati, OH) and ultracentrifugation at 100,000 ×g for 30 min. Membrane pellets were solubilized using a lysis buffer containing 1% DDM, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail (1:100) and phosphatase inhibitor cocktail 1 and 2 (each 1:100). Following ultracentrifugation at 100,000 ×*g* for 30 min, the soluble detergent lysates were incubated with 4 ml of WGA-bound agarose (Vector Laboratories, Burlingame, CA) for 6 h at 4 °C. The beads were extensively washed with column wash buffer containing 0.05% DDM, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. rKOR was eluted once overnight with column wash buffer containing 0.5 M *N*-acetylglucosamine at 4 °C and a second time for 1 h at room temperature.

4.8. Gel filtration chromatography

The WGA eluate (13 ml) was loaded onto a 2.6×90 cm column packed with Sephacryl S300 HR resin (Pharmacia, Piscataway, NJ). The column was eluted at a flow rate of 1.5 ml/min with 0.05% DDM, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. 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 20 μl aliquots of each fraction were assayed by western blotting using the anti-FLAG M1 antibody to detect rKOR.

The Sephacryl S300 column was calibrated using carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, beta-amylase, apoferritin and thyroglobulin as protein standards. Elution of the standards from the column was monitored by absorbance at 280 nm and verified by SDS/PAGE. A standard curve was generated by plotting the ratio of the elution volume of each protein to the column void volume versus the logarithm of the protein's molecular weight.

4.9. Anti-FLAG immunoaffinity chromatography

Gel filtration fractions containing rKOR were pooled and incubated with 1 ml of anti-FLAG M1 agarose beads for 6 h at 4 °C. The beads were extensively washed with column wash buffer containing 0.5% CHAPS, 50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. rKOR was eluted overnight at 4 °C with 20 mM EDTA in 0.5% CHAPS, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 10% glycerol. A second elution was collected after incubating for 1 h at room temperature. Both eluates were pooled for a total volume of 9 ml. CHAPS was used instead of DDM during the M1 wash and elution to minimize detergent concentration during the subsequent cutoff spin column as the CHAPS micelle is significantly smaller than the DDM micelle (6 kDa vs. 40–76 kDa). The volume of the M1 eluate was reduced to 0.2 ml using a 9000 MW cutoff spin column (Pierce Biotechnology) by centrifugation at 3000 ×*g* for 30 min. Due to concentration of CHAPS during the cutoff spin column, wash buffer containing 0.5% CHAPS, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 10% glycerol was added to reduce detergent concentration, bringing the final volume to 1 ml.

The concentrated M1 eluate was incubated at 4 °C overnight with 50 μl of anti-FLAG M2 beads and washed three times with the same column wash buffer. rKOR was eluted in 50 μl of 1× SDS loading dye. The sample was heated at 50 °C for 5 min and resolved using 12% SDS/PAGE. rKOR was visualized by staining the gel with E-Zinc protein stain (Pierce Biotechnology) and was excised from the gel with a sterile stainless steel scalpel. Gel fragments were diced into small pieces and transferred to a 1.5 ml microfuge tube.

4.10. In-gel digestion with trypsin and cyanogen bromide

Gel fragments were washed twice with 50 mM ammonium bicarbonate in 30% acetonitrile (ACN) prior to partial dehydration of the gel pieces with 80% ACN. Dithiothreitol (10 mM) in 25 mM ammonium bicarbonate was added and incubated for 30 min at 60 °C to reduce disulfide bonds. The dithiothreitol solution was removed and free cysteines were carbamidomethylated by adding 10 mM iodoacetamide in 25 mM ammonium bicarbonate and incubating in the dark at room temperature for 30 min. Gel fragments were washed with 50 mM ammonium bicarbonate in 30% ACN prior to dehydration as described above. Gel pieces were rehydrated with 25 mM ammonium bicarbonate containing 20 ng/μl sequencing grade trypsin (Promega, Madison, WI) and placed on ice for 45 min. Once rehydration was complete, trypsin digestion was carried out for 16 h at 37 °C. Peptides were extracted sequentially with 1% trifluoroacetic acid (TFA) in 40% ACN, 2% TFA in 40% ACN and 5% TFA in 80% ACN.

Following extraction of the tryptic peptides, cyanogen bromide digestion was conducted as described previously (Ball et al., 1998). Briefly, gel fragments were incubated in 100 mM cyanogen bromide (Sigma) in 50% ACN/50% TFA. The reaction was flushed with nitrogen and incubated in the dark at room

temperature for 24 h. Peptides were extracted sequentially with 2% TFA in 40% ACN and 5% TFA in 80% ACN and pooled.

4.11. *Peptide desalting*

Extracted tryptic peptides were dried in a speedvac and resuspended in 1% acetic acid/5% ACN. Peptides were subjected to titanium dioxide (TiO_2) chromatography using NuTips™ (Glygen, Columbia, MD) in an attempt to enrich for phosphopeptides. Peptides were eluted from the columns with 5% ammonium hydroxide, and following neutralization, were desalted using a C_4 ZipTip (Millipore, Billerica, MA) and eluted in 0.3% TFA/90% ACN. The TiO_2 flow-through fraction was also subjected to C_4 desalting. Peptides in the C_4 flow-through fractions were desalted using a C_{18} ZipTip (Millipore, Billerica, MA) and were eluted in 0.3% TFA/90% ACN.

For desalting of cyanogen bromide digested peptides, the ACN concentration was reduced to approximately 5% by adding 9 volumes of 0.1% TFA in H_2O. Peptides were then loaded onto a C_4 ZipTip, washed and eluted as described above.

4.12. *Mass spectrometry*

Desalted peptides were mixed with an equal volume of matrix consisting of 7 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma), 60% ACN, 0.1% TFA, 5 mM ammonium phosphate (Smirnov et al., 2004) and 50 fmol/μl Glu-fibrinopeptide and 50 fmol/μl ACTH fragment 18–39 (Anatrace, Maumee, OH) to serve as internal standards. Peptides were analyzed on a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA) in positive ion mode and spectra were analyzed using Data Explorer v4.5 (Applied Biosystems). MS peaks with a signal-to-noise ratio greater than 10 were screened against a theoretical digest of rKOR with a tolerance of 30 ppm using FindMod (http://www.expasy.org/tools/findmod/). Oxidized methionine (MSO) and carbamidomethyl (CAM) modification of cysteines by iodoacetamide were included as variable modifications during the search. rKOR peptides identified by MS were further analyzed by MS/MS to confirm their amino acid sequence. Significant MS/MS rKOR peptides were identified using MASCOT, v1.9 (Matrix Science, Boston, MA). Identification of HSP70 was performed by searching the MS/MS spectra against the Swiss-Prot database using MASCOT, v1.9 (Matrix Science).

4.13. *Detection of HSP70 in rKOR immunoprecipitates*

Cells were extracted with RIPA buffer containing 1% Nonidet P40 (NP40), 0.5% sodium deoxycholate, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol and protease inhibitor cocktail (Sigma, diluted 1:100). The detergent lysate was centrifuged at 16,000×*g* for 20 min, and the supernatant was recovered. Lysates (6 mg) were incubated with 50 μl of anti-FLAG M1 agarose beads for 6 h at 4 °C. The beads were extensively washed with RIPA buffer and rKOR was eluted in 1× SDS loading dye. Samples were resolved using 12% SDS/PAGE followed by western blotting. HSP70 was detected using an anti-HSP70 antibody (Assay Designs, Ann Arbor, MI) and goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma).

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