

Agonist-Promoted Lys63-Linked Polyubiquitination of the Human κ -Opioid Receptor Is Involved in Receptor Down-Regulation^[S]

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ABSTRACT

Ubiquitination of the human κ opioid receptor (hKOR) expressed in Chinese hamster ovary (CHO) cells was observed in the presence of the proteasomal inhibitor *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132) and enhanced by the agonists (–)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny) cyclohexyl] benzeneacetamide (U50,488H) and dynorphin A (Dyn A). The dominant-negative (DN) mutants GRK2-K220R and β -arrestin (319–418), but not dynamin I-K44A, reduced Dyn A-stimulated hKOR ubiquitination, and a phosphorylation-defective hKOR mutant (hKOR-S358N) did not undergo Dyn A-stimulated ubiquitination, indicating that hKOR ubiquitination is enhanced by receptor phosphorylation but not by receptor internalization. A hKOR mutant (hKOR-10 KR) in which all 10 intracellular Lys residues were changed to Arg showed greatly reduced basal and agonist-promoted receptor ubiquitination and substantially

decreased Dyn A-induced receptor down-regulation, without changing ligand binding affinity, receptor-G protein coupling, or receptor internalization or desensitization. The ubiquitination sites were further determined to be the three Lys residues in the C-terminal domain. The K63R ubiquitin mutant decreased Dyn A-induced hKOR ubiquitination and down-regulation, but the K48R mutant did not. Expression of HN-CYLD, a DN mutant of deubiquitination enzyme cylindromatosis tumor suppressor gene (CYLD) that breaks Lys63-linked polyubiquitin chain, increased Dyn A-induced hKOR ubiquitination and down-regulation. These results indicate that ubiquitinated hKOR after agonist treatment contains predominantly Lys63-linked polyubiquitin chains and ubiquitination of the hKOR involved in agonist-induced down-regulation.

Effects of opioid drugs and endogenous peptides are mediated by opioid receptors. There are at least three types of opioid receptors: μ , δ , and κ . The opioid receptors, belonging to the rhodopsin subfamily of the seven transmembrane domain receptor (7TMR) family, are coupled to pertussis toxin-sensitive G_i/G_o proteins, and activation of their effectors include adenylate cyclase, potassium and calcium channels, and mitogen-activated protein kinase pathways (for review, see Law et al., 2000). Activation of the κ opioid receptor

produces many effects, including analgesia, water diuresis, dysphoria, and antipruritic effects [see Liu-Chen (2004) for references].

After exposure to some agonists, the human κ opioid receptor (hKOR) expressed in Chinese hamster ovary (CHO) cells or human embryonic kidney 293 cells undergoes internalization (for review, see Liu-Chen, 2004) via G protein-coupled receptor kinase (GRK)-, arrestin-, and dynamin-dependent pathways that are likely to involve clathrin-coated vesicles (Li et al., 1999), similar to many other 7TMRs. After internalization, the receptor is recycled to plasma membranes or trafficked to lysosomes and proteasomes for degradation. We have demonstrated that EBP50/NHERF-1 interacts with the C terminus of the hKOR, probably in a protein

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ABBREVIATIONS: 7TMR, seven transmembrane domain receptor; GRK, G protein-coupled receptor kinase; β_2 -AR, β_2 -adrenergic receptor; CHO cells, Chinese hamster ovary cells; CHO-FLAG-hKOR, CHO cell lines stably expressing FLAG-hKOR; CHO-FLAG-hKOR-10KR, CHO cell lines stably expressing FLAG-hKOR-10KR; CYLD, cylindromatosis tumor suppressor gene; DN, dominant-negative mutant; Dyn A, dynorphin A (1–17); ER, Endoplasmic reticulum; FLAG-hKOR, Flag-tagged human κ opioid receptor; hKOR, human κ opioid receptor; hKOR-10KR, a FLAG-hKOR mutant in which all 10 intracellular lysines were replaced with arginines; NK-1, neurokinin-1 receptor; PAR, protease-activated receptor; U50,488H, (–)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny) cyclohexyl] benzeneacetamide; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; HA, hemagglutinin; HA-Ub, HA-ubiquitin; PBS, phosphate-buffered saline; UBPY, ubiquitin-specific protease Y.

complex, and this association seems to serve as a signal for internalized hKOR to be sorted to the recycling pathway (Li et al., 2002b). In contrast, the signals that direct internalized receptors to degradation pathways have not been fully elucidated. Two biochemical mechanisms are associated with sorting of internalized 7TMRs to lysosomes: proteins associated with receptors and ubiquitination of the receptor. Proteins associated with 7TMRs include sorting nexin 1 for protease-activated receptor (PAR) 1 (Wang et al., 2002) and G protein-coupled receptor-associated sorting protein for the δ opioid receptor (Whistler et al., 2002). Ubiquitination of β_2 -adrenergic (β_2 -AR) (Shenoy et al., 2001), chemokine CXCR4 (Marchese and Benovic, 2001; Marchese et al., 2003), V2 vasopressin (Martin et al., 2003), and neurokinin-1 (NK-1) (Cottrell et al., 2006) receptors and PAR2 (Jacob et al., 2005) is important for sorting of the internalized receptors to lysosomes; however, ubiquitination seems not to play a role in the trafficking of the δ opioid receptor (Chaturvedi et al., 2001; Tanowitz and von Zastrow, 2002).

Ubiquitination is an important post-translational modification, by which ubiquitin, a 76-amino acid polypeptide, is conjugated to a substrate protein through the formation of an isopeptide bond between the ϵ amino group of Lys residues in target proteins and the C-terminal glycine of ubiquitin (for review, see Herskho and Ciechanover, 1998). Ubiquitination is catalyzed by three enzymes acting in tandem: an E1, a ubiquitin-activating enzyme, an E2, a ubiquitin-carrying enzyme; and an E3, a ubiquitin ligase. There are approximately 400 E3 ubiquitin ligases, which interact directly with the target protein or indirectly through an adapter protein and confer specificity. For mono- and multimono-ubiquitination, single ubiquitin molecules are conjugated to one or more Lys residues. Because ubiquitin contains Lys residues, multiple ubiquitins can be linked to one or more Lys residues of the substrate sequentially, termed polyubiquitination. Only a small number of 7TMRs have been examined for ubiquitination, and both mono- and polyubiquitination have been reported. Ubiquitination of 7TMRs has been shown to be involved in agonist-induced internalization, ER-associated degradation of misfolded receptors, and sorting of internalized receptor to lysosomes for degradation (for review, see Shenoy, 2007).

Our previous observations that U50,488H-induced down-regulation of the hKOR expressed in CHO cells was partially reduced by the proteasome inhibitor MG132 or the lysosome inhibitor chloroquine (and almost totally abolished by the combination of the two) indicate that lysosomes as well as proteasomes are involved in agonist-induced down-regulation of the hKOR (Li et al., 2000).

In this study, we found that the hKOR expressed in CHO cells was ubiquitinated after exposure to an agonist. We examined whether agonist-induced ubiquitination of the hKOR was regulated by receptor phosphorylation and internalization. We then determined the functional significance of ubiquitination of the hKOR by generating a hKOR mutant that was not ubiquitinated and comparing the mutant with the wild type in receptor function, trafficking, and regulation. We also tested the involvement of Lys63- or Lys48-linked polyubiquitination in the ubiquitination and regulation of the hKOR.

Materials and Methods

Materials. [35 S]GTP- γ S (~1250 Ci/mmol) and [3 H]diprenorphine (~58 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Dyn A was purchased from Phoenix Pharmaceuticals (Belmont, CA). U50,488H and naloxone were provided by the National Institute on Drug Abuse (Bethesda, MD). The following reagents were bought from the indicated companies: rabbit polyclonal antibody against the FLAG epitope (F7425), anti-FLAG M2 affinity gel, peroxidase-conjugated goat anti-rabbit IgG, chloroquine, and MG132 from Sigma Co. (St. Louis, MO); polyclonal rabbit anti-HA antibody from Zymed Co. (South San Francisco, CA); anti-HA monoclonal antibody (HA-11) from Covance (Cumberland, VA), Alexa Fluor 488-conjugated goat anti-mouse IgG from Invitrogen (Carlsbad, CA); and SuperSignal West Pico chemiluminescent substrate kit from Pierce Chemical (Rockford, IL). The HA-ubiquitin (HA-Ub) expression plasmid was kindly provided by Dr. D. Bohmann of the University of Rochester. Dynamin I-K44A, β -arrestin (319–418), and GRK2-K220R were gifts from Dr. Jeffrey Benovic of Thomas Jefferson University; HA-Ub, HA-Ub-K63R, HA-Ub-K48R, CYLD, and HN-CYLD were from Dr. Marie Wooten of Auburn University.

Construction of FLAG-hKOR-10KR cDNA. cDNA encoding human κ opioid receptor with N-terminal FLAG epitope (FLAG-hKOR) in pcDNA3 was generated previously (Xu et al., 2000). A FLAG-hKOR mutant in which all 10 predicted intracellular lysines (at positions 89, 91, 165, 174, 176, 254, 265, 338, 349, and 378) were replaced with arginines was generated using point mutations and the overlap polymerase chain reaction method with the FLAG-hKOR in pcDNA3 as the template. Mutated cDNA (named FLAG-hKOR-10KR) was cloned into pcDNA3, and DNA sequence determination was performed to ensure correct generations of all mutations and no unwanted mutations.

Stable Expression of FLAG-hKOR-10KR in CHO Cells. Clonal CHO cells stably transfected with the FLAG-hKOR-10KR cDNA (CHO-FLAG-hKOR-10KR) were established, and a cell line with a receptor expression level similar to that of CHO cells stably transfected with the FLAG-hKOR cDNA (CHO-FLAG-hKOR) (Li et al., 2002a) was selected for this study. CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% fetal calf serum, 0.1 mg/ml G-418 (Geneticin), 100 units/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere consisting of 5% CO₂/95% air at 37°C.

Ubiquitination of FLAG-hKOR. The experiment was performed according to a modification of our published procedure (Li et al., 2002b). CHO cells in 100-mm dishes were grown to ~90% confluence and transiently transfected with 8 μ g of HA-Ub and 10 μ g of FLAG-hKOR, FLAG-hKOR-10KR, FLAG-hKOR-S358N, or the vector pcDNA3. CHO-FLAG-hKOR cells were transiently transfected with 8 μ g of HA-Ub and 10 μ g of dynamin I-K44A, β -arrestin (319–418), or GRK2-K220R. Approximately 2 days later, cells were treated with 50 μ M MG132 for 3 h, and then 1 μ M Dyn A was added for another 30 min. Then cells were lysed, solubilized with solubilization buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, and 20 μ M *N*-ethylmaleimide, pH 7.4) for 1 h at 4°C and centrifuged at 100,000g for 1 h. Solubilized materials were incubated for overnight at 4°C with anti-FLAG M2 affinity gel to immunoprecipitate FLAG-hKOR or with HA-11 to immunoprecipitate ubiquitinated hKOR. The immunoprecipitated materials were washed, and Laemmli sample buffer was added with 100 mM dithiothreitol (DTT). For removal of proteins immunoprecipitated with FLAG-hKOR, 50 μ l of 2% SDS sample buffer was added to immunoprecipitated materials. The samples were then incubated with 950 μ l of solubilization buffer and 50 μ l of anti-FLAG M2 affinity gel for 1 h at 4°C. Then the immunoprecipitated materials were washed and Laemmli sample buffer was added with 100 mM DTT. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred

onto polyvinylidene difluoride membranes (Immobilon; Millipore, Billerica, MA). Membranes were blotted with a rabbit polyclonal antibody against HA or rabbit polyclonal antibody against the FLAG epitope followed by goat anti-rabbit polyclonal IgG conjugated with horseradish peroxidase and reacted with ECL western blotting detection reagents. Images were captured by use of a FujiFilm LAS-1000 plus system (FujiFilm, Tokyo, Japan).

Quantitation of Receptor Down-Regulation by Immunoblotting. Immunoblotting was performed to examine the level of the FLAG-hKOR proteins as described previously (Chen et al., 2006b). In brief, CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells in 12-well plates were treated with or without 1 μ M Dyn A for 4 h at 37°C. Cells were solubilized with 2 \times Laemmli sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinylidene difluoride membranes. Immunoblotting was performed with a rabbit polyclonal antibody against FLAG, goat anti-rabbit polyclonal IgG conjugated with horseradish peroxidase and ECL western blotting detection reagents. The results were detected with a Fuji Film LAS-1000 system and quantitated by ImageGauge (version 4.1; Fuji PhotoFilm Co. Ltd.).

Quantitation of cell surface receptors by fluorescence flow cytometry and determination of receptor internalization and down-regulation were performed according to our published procedure (Li et al., 2003). In brief, CHO-FLAG-hKOR or CHO-FLAG-hKOR-10KR cells grown in 12-well plates were treated with or without 10 μ M U50,488H for 30 min (for internalization) or 1 μ M Dyn A for 4 h (for down-regulation) at 37°C. Cells were washed three times with ice-cold PBS buffer (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 68 mM NaCl), and lifted with PBS buffer containing 0.5 mM EDTA. Cells were incubated with M1 anti-FLAG antibody (1 μ g/ml) in 300 μ l of Opti-MEM I reduced serum medium (Invitrogen) containing 1 mM CaCl₂ for 45 min at 4°C. After three washes with PBS buffer, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1 μ g/ml) in 300 μ l of Opti-MEM I reduced serum medium containing 1 mM CaCl₂ for 45 min at 4°C. Cells were washed three times with ice-cold PBS buffer containing 1 mM CaCl₂, and then re-suspended with 300 μ l of PBS buffer. Immunoreactivity of cell surface receptors was quantitated by fluorescence flow cytometry (FACScan; BD Biosciences, San Jose, CA). Reduction in cell surface immunofluorescence was used to as the measures of receptor internalization and down-regulation.

κ Opioid Receptor Binding. Membrane preparations and receptor binding of CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells was conducted with [³H]diprenorphine in 50 mM Tris-HCl buffer as described previously (Li et al., 1999). Naloxone (10 μ M) was used to define nonspecific binding. Saturation experiments were performed with various concentrations of [³H]diprenorphine (ranging from 0.02 to 2 nM) to determine total receptor number. Competitive inhibition of [³H]diprenorphine binding was performed with [³H]diprenorphine at a concentration \sim 0.3 nM and various concentrations of U50,488H or Dyn A to determine the affinity of their binding to receptor. Binding was conducted at 25°C for 60 min in duplicate in a volume of 1 ml with approximately 15 μ g of membrane protein. Bound and free [³H]diprenorphine were separated by rapid filtration under reduced pressure over GF/B filters presoaked with 0.2% polyethylenimine and 0.1% bovine serum albumin in 50 mM Tris-HCl, pH 7.4, for 1 h. Binding data were analyzed with nonlinear regression in Prism 3.0 (GraphPad Software, Inc., San Diego, CA) and the data fitted a one-site binding curve.

[³⁵S]GTP γ S Binding and U50,488H-Induced Desensitization. Membrane preparations and [³⁵S]GTP γ S binding were performed according to Li et al. (2002a). In brief, cells were treated without or with 1 μ M U50,488H for 30 min and washed three times with ice-cold PBS buffer. Then, the cells were pelleted and ice-cold lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10 mM sodium fluoride, and 10 mM tetrasodium pyrophosphate, pH 7.4) was added. Cell suspension was passed through a 1-ml, 29-gauge, 3/8" syringe

needle five times and then centrifuged. Pellets were re-suspended in 50 mM Tris-HCl buffer, pH 7.4, passed through the syringe needle and centrifuged, and the processes were repeated one more time. Membranes were suspended in 50 mM Tris-HCl buffer, pH 7.4, protein contents were determined by the BCA method, and membranes were frozen at -80°C until use. Approximately 10 μ g of membrane protein was incubated without or with different concentration of U50,488H, 15 μ M GDP, and 0.2 nM [³⁵S]GTP γ S in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 0.1% BSA, pH 7.4) in a final volume of 0.5 ml. After 60-min incubation at 30°C, bound and free [³⁵S]GTP γ S were separated by filtration with GF/B filters. Radioactivity on filters was determined by liquid scintillation counting.

Statistical Analysis. For comparison of multiple groups, data were analyzed with analysis of variance to determine whether there were significant differences among groups using Prism 3.0 (GraphPad Software, Inc.). If so, Dunnett's post hoc test was performed to determine whether there was significant difference between the control and each treatment group. For comparison of two groups, two-tailed Student's *t* test was performed. *P* < 0.05 was the level of significance in all statistical analyses.

Results

The hKOR Was Ubiquitinated and Agonists Enhanced Its Ubiquitination. CHO cells were transiently transfected with HA-Ub and FLAG-hKOR or the vector pcDNA3 and treated with 50 μ M MG132 and then with or without 1 μ M U50,488H or 1 μ M Dyn A for 30 min. Ubiquitination of the receptor was examined by immunoprecipitation of FLAG-hKOR with anti-FLAG antibody and immunoblotting with anti-HA antibody (Fig. 1A). Alternatively, ubiquitinated FLAG-hKOR was detected by immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-FLAG antibody (Fig. 1B). As shown in Fig. 1, compared with pcDNA-transfected control, the hKOR was ubiquitinated in the presence of MG132. There was no detectable ubiquitination of hKOR without MG132 treatment (data not shown). Incubation with U50,488H or Dyn A increased ubiquitination of the receptor (Fig. 1). We also carried out immunoprecipitation under conditions that removed associated proteins. The first immunoprecipitation was performed with anti-FLAG antibody, and the immunoprecipitated materials were denatured with 2% SDS to dissociate proteins immunoprecipitated with hKOR. Triton X-100 was added to surround SDS, and the second immunoprecipitation was then conducted with anti-FLAG antibody. Immunoblotting was performed with anti-HA antibody. Under these conditions, we also found that Dyn A induced hKOR ubiquitination (Fig. 1C). It seems that ubiquitinated hKOR represents a small percentage of total receptors.

FLAG-hKOR expressed in CHO cells migrated as two bands with molecular mass of 45 and 55 kDa (Fig. 1), similar to our previous findings (Chen et al., 2006a). In addition, the 55-kDa band represents the fully glycosylated FLAG-hKORs (mature form), most of which locates in plasma membranes, whereas the 45-kDa band represents *N*-linked glycosylated high-mannose intermediates (immature form), which mainly locate in ER and *cis*-Golgi (Chen et al., 2006a). Treatment with 1 μ M U50,488H or 1 μ M Dyn A for 30 min did not change the level of either protein band (Fig. 1, A and C).

Ubiquitination of the hKOR Was Enhanced by Receptor Phosphorylation but Not by Receptor Internalization. To investigate whether agonist-induced ubiquitina-

tion of the hKOR requires receptor phosphorylation and internalization, we examined effects of DN mutants of GRK2, β -arrestin, and dynamin I. Transient cotransfection of CHO-FLAG-hKOR cells with HA-Ub and GRK2-K220R or β -arrestin (319–418), but not dynamin I-K44A, significantly reduced Dyn A-stimulated hKOR ubiquitination (Fig. 2, A and B). However, the basal ubiquitination of the hKOR was not

affected by expressions of DN mutants of GRK2, β -arrestin, and dynamin I (see Supplemental Data 1). To further examine the role of phosphorylation, we tested whether a phosphorylation-defective hKOR mutant (hKOR-S358N) (Li et al., 2002a) underwent ubiquitination. CHO cells were transiently transfected with HA-Ub and FLAG-hKOR or FLAG-hKOR-S358N to similar receptor expression levels, and re-

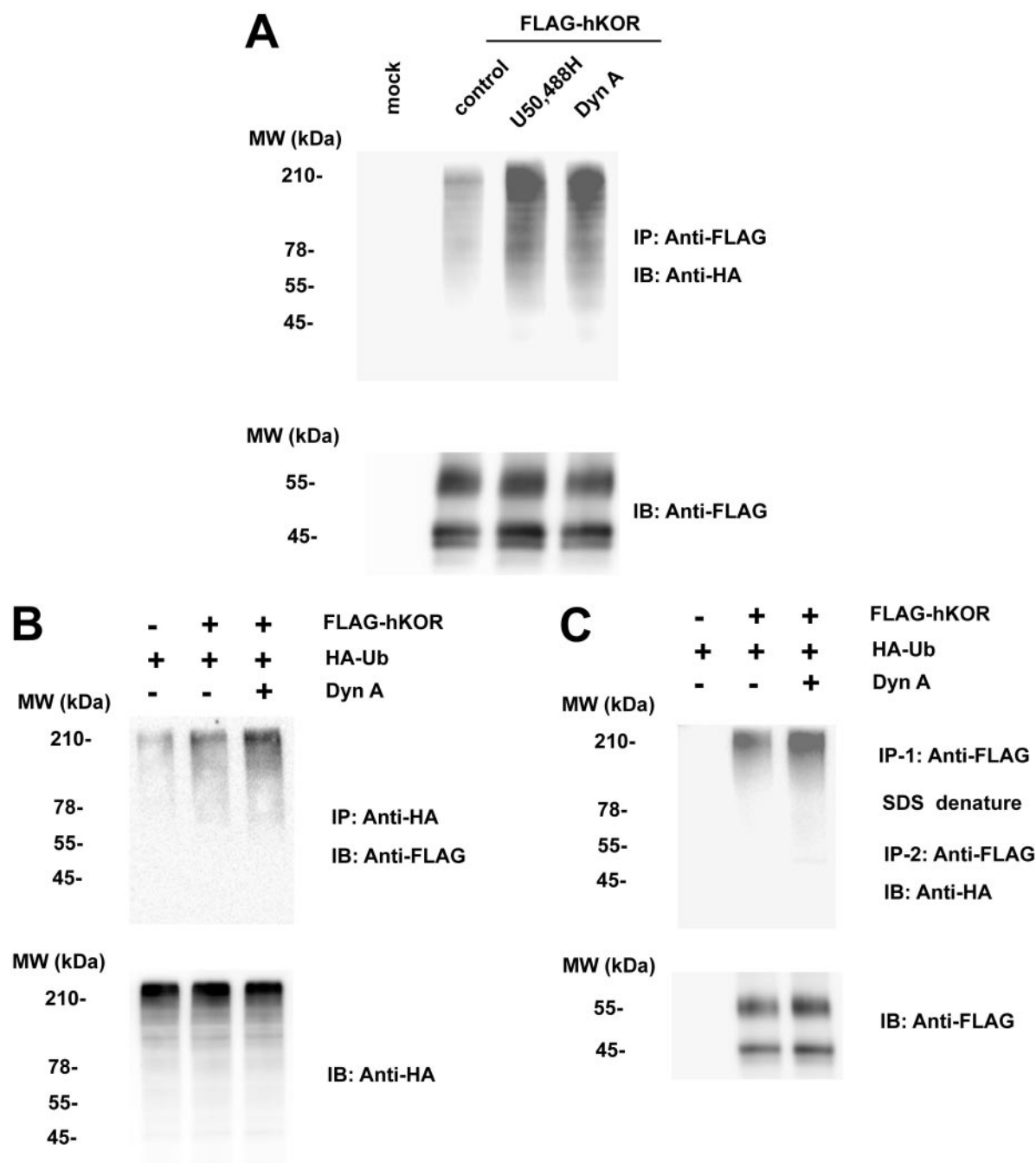


Fig. 1. Ubiquitination of the hKOR was enhanced by U50,488H and Dyn A. CHO cells were transiently transfected with HA-Ub and FLAG-hKOR or the vector pcDNA3. Approximately 2 days later, cells were treated with 50 μ M MG132 for 3 h and then without or with 10 μ M U50,488H or 1 μ M Dyn A for another 30 min. A, cells were lysed, solubilized, and immunoprecipitated with anti-FLAG M2 affinity gel, and Western blot was performed using rabbit anti-HA antibody (top) or rabbit anti-FLAG antibody (bottom) as described under *Materials and Methods*. B, cells were lysed, solubilized, and immunoprecipitated with anti-HA monoclonal antibody, and Western blot was performed using rabbit anti-FLAG antibody (top) or rabbit anti-HA antibody (bottom) as described under *Materials and Methods*. C, cells were lysed, solubilized and immunoprecipitated with anti-FLAG M2 affinity gel, treated with 2% SDS Laemmli sample buffer followed by addition of excess Triton X-100 and then immunoprecipitation with anti-FLAG M2 affinity gel again. Immunoblotting was performed using rabbit anti-HA antibody (top) or rabbit anti-FLAG antibody (bottom) as described under *Materials and Methods*. Each figure represents one of three independent experiments performed with similar results.

ceptor ubiquitination was detected. The hKOR-S358N mutant showed greatly reduced Dyn A (1 μ M)-stimulated ubiquitination, compared with the wild type (Fig. 2C). These results indicate that agonist-stimulated ubiquitination of the hKOR is enhanced by receptor phosphorylation and association of phosphorylated receptor with clathrin via β -arrestin but not by receptor internalization. Thus, hKOR ubiquitination occurs to receptors mostly in plasma membranes.

Mutations of All 10 Intracellular Lys Residues to Arg (10KR) Greatly Reduced Ubiquitination of the hKOR.

To examine the functional importance of hKOR ubiquitination, we generated a hKOR mutant, named hKOR-10KR, in which all 10 Lys residues in the intracellular loops and C-terminal domain, the potential ubiquitination sites, were mutated to Arg. Figure 3A shows a schematic representation of the hKOR and the 10 intracellular Lys residues denoted by asterisks. CHO cells were transiently transfected with HA-Ub and FLAG-hKOR or FLAG-hKOR-10KR, and agonist-stimulated receptor ubiquitination was examined. 10KR mutations greatly reduced basal and Dyn A-stimulated receptor ubiquitination, compared with the wild type (Fig. 3B). These results indicate that ubiquitination occurs on Lys residues in intracellular domains of the hKOR.

10KR Mutations of the hKOR Did Not Change the Binding Affinity for [3 H]Diprenorphine, U50,488H, and Dyn A, Receptor-G Protein Coupling and Agonist-Induced Desensitization. CHO cells stably expressing FLAG-hKOR-10KR were established, and one clonal cell line with receptor expression level similar to that of CHO-FLAG-hKOR cells was selected for the subsequent experiments. Saturation binding of [3 H]diprenorphine binding and competitive inhibition of [3 H]diprenorphine binding by

U50,488H and Dyn A were performed on membranes prepared from both cell lines (Table 1). The wild type and the 10KR mutant had similar K_d values for [3 H]diprenorphine and comparable K_i values for U50,488H and Dyn A (Table 1). These results indicate that mutations of all the intracellular Lys to Arg did not overtly change the receptor binding pocket.

U50,488H-stimulated [35 S]GTP γ S binding to membranes of both CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells was examined with or without pretreatment with 1 μ M U50,488H (Fig. 4). Without U50,488H pretreatment, the EC_{50} and E_{max} values of U50,488H in stimulating [35 S]GTP γ S binding in both cell lines are similar, indicating that the 10KR mutations do not affect receptor coupling to G protein signaling. Pretreatment with U50,488H reduced the E_{max} value and increased the EC_{50} value compared with untreated controls ($P < 0.05$ for both) and hence desensitized both the wild type and 10KR mutant (Fig. 4). After pretreatment with U50,488H, EC_{50} and E_{max} values were not different between the wild type and 10KR mutant ($P > 0.05$). The result demonstrates that mutations of the 10 Lys residues to Arg did not affect U50,488H-induced receptor desensitization.

10KR Mutations of the hKOR Decreased Agonist-Induced Receptor Down-Regulation. Both CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells were treated with or without 1 μ M Dyn A for 4 h or indicated times, and receptor down-regulation was determined. We have demonstrated previously that agonist-promoted down-regulation of the hKOR reaches a plateau at 4 h (Zhu et al., 1998). In addition, Dyn A causes a higher degree of hKOR down-regulation than U50,488H because U50,488H and other

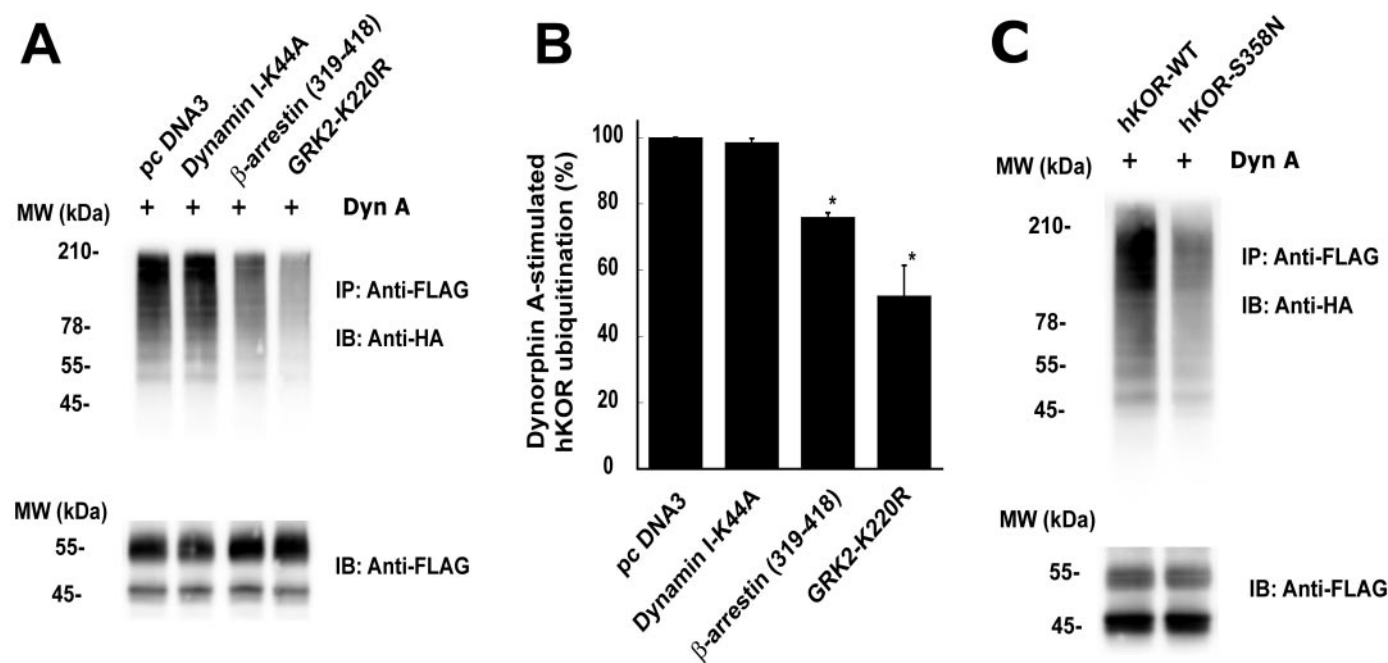


Fig. 2. Ubiquitination of the hKOR was regulated by receptor phosphorylation but not by receptor internalization. A and B, CHO-FLAG-hKOR cells were transiently transfected with HA-Ub and FLAG-hKOR or FLAG-hKOR-S358N to similar receptor expression levels. A and C, approximately 2 days later, cells were treated with 50 μ M MG132 for 3 h, and 1 μ M Dyn A was then added for another 30 min. Cells were lysed, solubilized, and immunoprecipitated with anti-FLAG M2 affinity gel, and Western blot was performed using rabbit anti-HA antibody (top) or rabbit anti-FLAG antibody (bottom) as described under *Materials and Methods*. Each figure represents one of three independent experiments performed with similar results. B, quantitation of results from A. Densitometric analysis was performed using the OptiQuant software. Each point represents the mean \pm S.E.M. of three independent experiments. *, $P < 0.05$, compared with the pcDNA3 control group by two-tailed Student's t test.

levels are significantly lower for the 10 KR mutant, compared with the wild type (Fig. 5B). The results indicate that ubiquitination of hKOR is involved in agonist-induced down-regulation. However, because the 10KR mutant still underwent down-regulation, agonist-promoted polyubiquitination is not required for hKOR down-regulation.

Pretreatment with the lysosome inhibitor chloroquine (50 μ M) for 1 h partially blocked Dyn A-induced 10KR down-regulation (see Supplemental Data 2). These data indicate

Receptor expression levels and ligand binding affinities of the hKOR-WT and hKOR-10KR

Membranes of CHO cells stably expressing the FLAG-hKOR or FLAG-hKOR-10KR were prepared and B_{\max} and K_d values of [3 H]diprenorphine and K_i values of U50,488H and Dyn A were determined as described under *Materials and Methods*. Each value represents the mean \pm S.E.M. of three independent experiments.

	$[^3\text{H}]\text{Diprenorphine}$		K_i	
	B_{max}	K_d	U50,488H	Dyn A
	<i>pmol/mg protein</i>	<i>nM</i>	<i>nM</i>	<i>nM</i>
hKOR-WT	1.71 ± 0.17	0.12 ± 0.01	1.45 ± 0.14	0.10 ± 0.01
hKOR-10KR	2.20 ± 0.17	0.14 ± 0.02	1.64 ± 0.26	0.11 ± 0.02

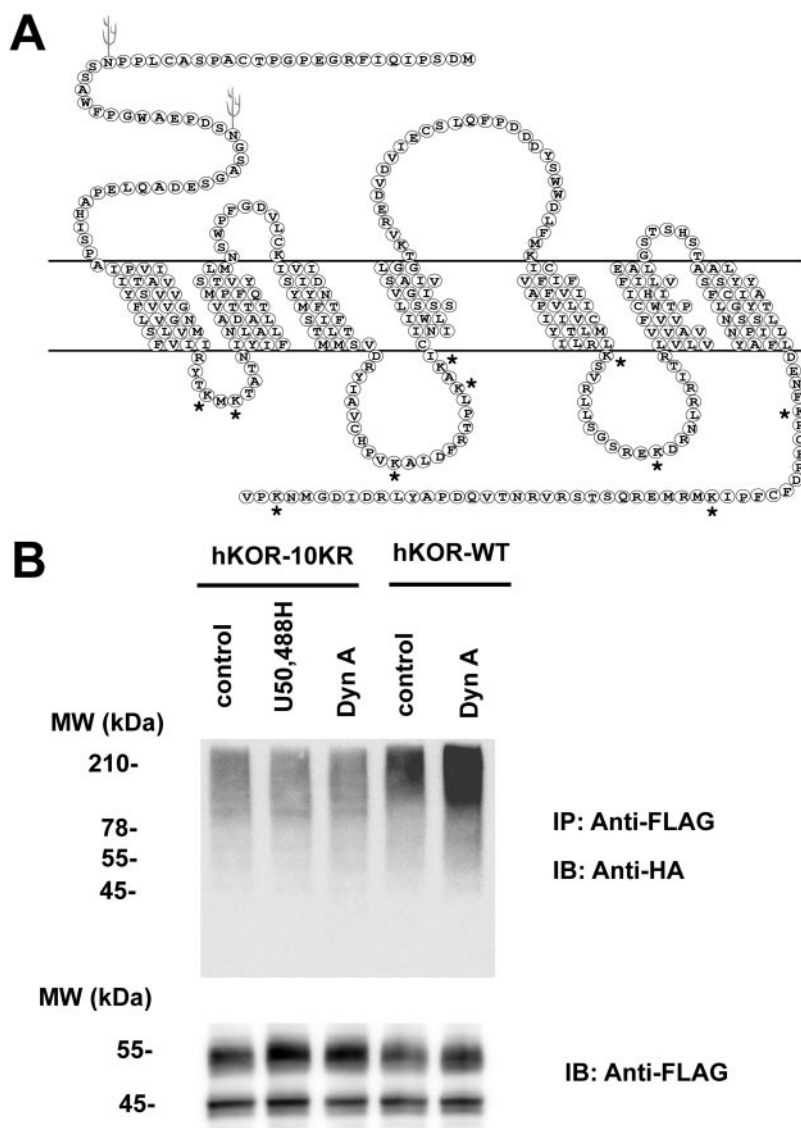
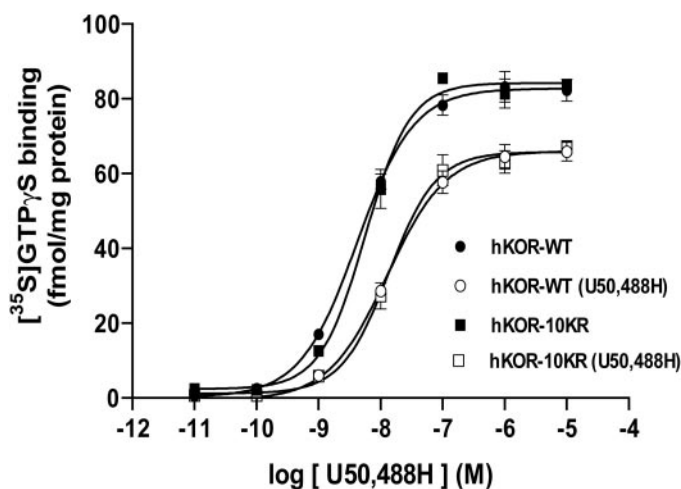


Fig. 3. Mutations of the intracellular Lys residues abolished ubiquitination of the hKOR. A, schematic representation of the hKOR and the intracellular Lys residues mutated. A hKOR mutant (hKOR-10 KR) was generated by changing all 10 Lys residues to Arg (at positions 89, 91, 165, 174, 176, 254, 265, 338, 349, and 378) denoted by asterisks in the intracellular loops and C-terminal domain. B, CHO cells were transiently transfected with HA-Ub and FLAG-hKOR or FLAG-hKOR-10KR 3 as indicated. Approximately 2 days later, cells were treated with 50 μ M MG132 for 3 h and then without or with 1 μ M U50,488H or 1 μ M Dyn A for another 30 min. The cells were lysed, solubilized, and immunoprecipitated with anti-FLAG M2 affinity gel, and Western blot was performed using rabbit anti-HA antibody (top) or rabbit anti-FLAG antibody (bottom) as described under *Materials and Methods*. Each figure represents one of three independent experiments performed with similar results.



	EC ₅₀ (nM)		E _{max} (fmol/mg protein)	
	Control	U50,488H	Control	U50,488H
hKOR-WT	4.18 ± 0.17	12.8 ± 0.59 *	82.8 ± 0.46	66.0 ± 0.50 *
hKOR-10KR	5.65 ± 0.85	13.6 ± 1.71 *	84.3 ± 1.80	65.8 ± 1.47 *

Fig. 4. Mutation of the hKOR ubiquitination sites did not change agonist-induced [³⁵S]GTP γ S binding and desensitization. CHO-FLAG-hKOR or CHO-FLAG-hKOR-10KR cells were incubated without or with 1 μ M U50,488H for 30 min and washed. Membranes were prepared, and U50,488H-stimulated [³⁵S]GTP γ S binding was determined as described under *Materials and Methods*. The EC₅₀ and E_{max} values were showed in the table. Each value represents the mean \pm S.E.M. of three independent experiments. *, $P < 0.05$, compared with the control group by two-tailed Student's t test.

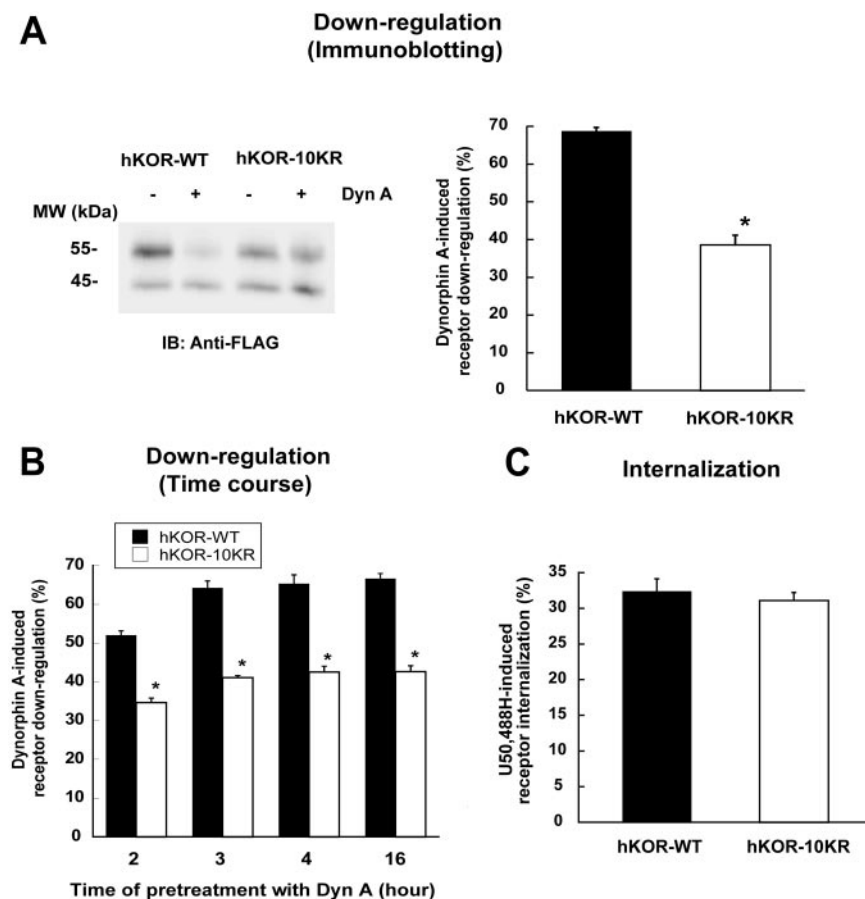
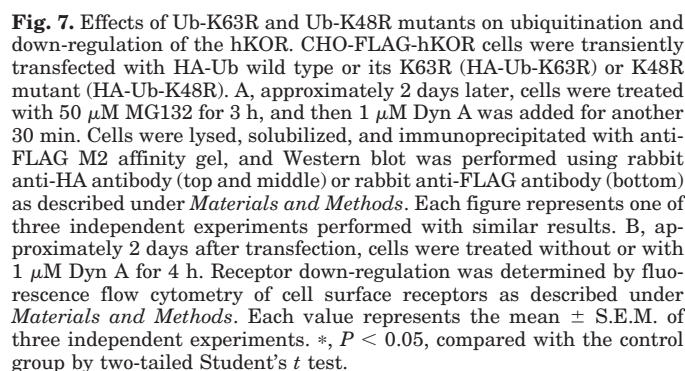


Fig. 5. Mutation of the hKOR ubiquitination sites decreased agonist-induced receptor down-regulation but did not affect agonist-induced internalization. CHO-FLAG-hKOR or CHO-FLAG-hKOR-10KR cells were treated without or with 1 μ M Dyn A for 4 h A, or indicated time periods for down-regulation B, or with 10 μ M U50,488H for 30 min for internalization. Receptor down-regulation was determined by immunoblotting with anti-FLAG antibody followed by quantitation of the 55-kDa form (A) or fluorescence (C) flow cytometry of cell surface receptors as described under *Materials and Methods* (B). C, for agonist-induced internalization, cell surface receptors were determined using the fluorescence flow cytometry assay. Each value represents the mean \pm S.E.M. of three independent experiments. *, $P < 0.05$, compared with the control group by two-tailed Student's t test.

that part of the unubiquitinated hKOR-10 KR is trafficked to lysosomes for degradation.

10KR Mutations of the hKOR Did Not Alter Agonist-Induced Receptor Internalization or Recycling after Agonist Removal. Reduced Dyn A-induced down-regulation observed for the 10KR mutant may be attributed to decreased receptor internalization. We thus examined whether 10KR mutations affected internalization. We previously demonstrated that U50,488H and Dyn A promoted similar levels of internalization of the hKOR (Li et al., 2003; Chen et al., 2006b). Both CHO-FLAG-hKOR and CHO-FLAG-hKOR-10KR cells were incubated without or with 10 μ M U50,488H for 30 min, and receptor internalization was determined using the fluorescence flow cytometry assay. The extent of receptor internalization induced by U50,488H were similar for the wild type and hKOR-10KR (Fig. 5C), indicating that receptor ubiquitination is not required for agonist-induced internalization of the hKOR. In addition, the recycling rates of the hKOR and 10KR mutant after removal of U50,488H were not significantly different as determined by the fluorescence flow cytometry assay (see Supplemental Data 3).

Ubiquitination of the hKOR Occurred Predominantly at Lys338, Lys349, and Lys378 in the C-Terminal Domain. To narrow down which intracellular Lys residues of the hKOR are important in receptor ubiquitination, we mutated the three Lys residues (Lys338, Lys349, and Lys378) in the C-terminal domain of the hKOR to Arg to get the hKOR-3KR mutant. The hKOR-3KR mutant, when transfected into CHO cells, showed greatly decreased agonist-induced ubiquitination, compared with wild type (Fig. 6). We then generated a CHO cell line



Effects of the HN-CYLD Mutant on Agonist-Induced Ubiquitination and Down-Regulation of the hKOR. To further examine whether the hKOR undergoes Lys63-linked polyubiquitination, we studied effect of the DN mutant of deubiquitination enzyme cylindromatosis tumor suppressor gene (CYLD), HN-CYLD, on Dyn A-stimulated ubiquitination of the hKOR. CYLD belongs to the family of ubiquitin-specific hydrolases, which are deubiquitinating cysteine proteases (Bignell et al., 2000), and it acts on Lys63-linked, but not Lys48-linked, polyubiquitin chains (Kovalenko et al., 2003; Trompouki et al., 2003). Transient expression of HN-CYLD greatly increased Dyn A-induced hKOR ubiquitination (Fig. 8A), indicating that the hKOR undergoes K63-linked polyubiquitination. Expression of HN-CYLD in FLAG-

hKOR-CHO cells reduced Dyn A-induced down-regulation of the hKOR (Fig. 8B). However, expression of wild type CYLD did not affect ubiquitination or down-regulation (data not shown). These results indicate that K63-linked polyubiquitination of the hKOR plays an important role in agonist-induced receptor down-regulation.

Discussion

The data reported herein provide evidence that hKOR undergoes agonist-dependent ubiquitination, and its ubiquitination regulates, but is not required for, sorting of internalized receptor to degradation pathway. Agonist-promoted ubiquitination of the hKOR, which occurs at Lys residues in the C-terminal domain of the hKOR, is enhanced by hKOR phosphorylation but unchanged by receptor internalization. In addition, agonist-promoted polyubiquitination of the hKOR occurs predominantly as Lys63-linked polyubiquitin chains. To the best of our knowledge, this is the first report that Lys63-linked polyubiquitination of a 7TMR is involved in its down-regulation.

Ubiquitination of the hKOR Regulates, but Is Not Required for, Agonist-Induced hKOR Down-Regulation. Ubiquitination of the hKOR was increased substantially after agonist stimulation. The rapid occurrence of ubiquitination of the hKOR after agonist incubation (30 min) is similar to that reported for several 7TMRs, including the β_2 -AR (within 15 min) (Shenoy et al., 2001). As shown in several figures, it is most likely that the hKOR is polyubiquitinated because ubiquitinated hKOR is of high molecular weight.

Mutations of the all intracellular Lys residues to Arg greatly reduced Dyn A-induced receptor ubiquitination and down-regulation (Fig. 5, A and B), but did not affect receptor internalization, indicating that intracellular Lys residues and/or ubiquitination of hKOR plays an important role in sorting of internalized hKOR for degradation. We showed that agonist-promoted down-regulation of the hKOR involved trafficking of the receptor from early endosomes to late endosomes to lysosomes (Li et al., 2000). The results indicate that ubiquitination of the hKOR is involved in targeting the receptor from endosomes to lysosomes. These results are consistent with those on several 7TMRs, including β_2 -AR (Shenoy et al., 2001), chemokine CXCR4 (Marchese and Benovic, 2001; Marchese et al., 2003), V2 vasopressin (Martin et al., 2003), NK-1 (Cottrell et al., 2006) and PAR2 (Jacob et al., 2005), but not those on the δ opioid receptor (Tanowitz and von Zastrow, 2002) and the complex of calcitonin receptor-like receptor and receptor activity-modifying protein 1 (Cottrell et al., 2007). Sorting of CXCR4 from endosomes to lysosomes requires Hrs and Vps4, an AAA ATPase (ATPases Associated with diverse cellular Activities); the latter is involved in regulating the ubiquitination status of both CXCR4 and Hrs (Marchese et al., 2003).

The 10KR mutations of FLAG-hKOR eliminated agonist-promoted ubiquitination and reduced, but did not abolish, Dyn A-induced down-regulation. Similar results were reported for the Lys-to-Arg mutants of the V2 vasopressin (Martin et al., 2003) and NK-1 (Cottrell et al., 2006) receptors. These results suggest that ubiquitination of these receptors plays a regulatory, but not a mandatory, role for trafficking of the receptors in multivesicular bodies en route

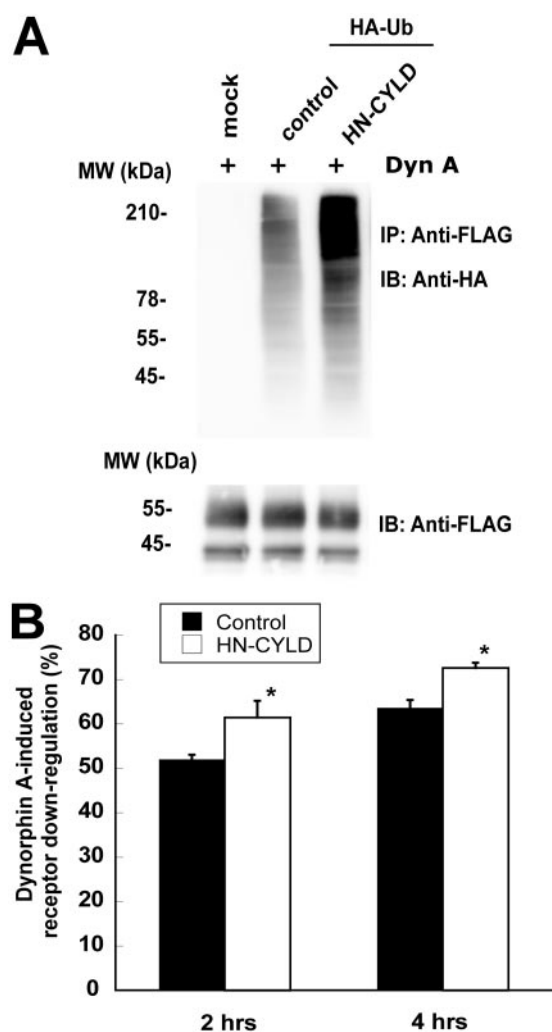


Fig. 8. Effect of HN-CYLD mutant on ubiquitination and down-regulation of the hKOR. CHO-FLAG-hKOR cells were transiently transfected with HA-Ub and HN-CYLD or pcDNA3. A, approximately 2 days later, cells were treated with 50 μ M MG132 for 3 h and then 1 μ M Dyn A was added for another 30 min. Cells were lysed, solubilized, and immunoprecipitated with anti-FLAG M2 affinity gel, and Western blot was performed using rabbit anti-HA antibody (top) or rabbit anti-FLAG antibody (bottom) as described under *Materials and Methods*. Each figure represents one of three independent experiments performed with similar results. B, approximately 2 days after transfection, cells were treated without or with 1 μ M Dyn A for 2 or 4 h. Receptor down-regulation was determined by fluorescence flow cytometry as described under *Materials and Methods*. Each value represents the mean \pm S.E.M. of three independent experiments. *, $P < 0.05$, compared with the control group by two-tailed Student's t test.

to lysosomes for degradation. For some 7TMRs, ubiquitination is essential. Unubiquitinated mutants of β_2 -AR, PAR2, and CXCR4 were not down-regulated after agonist treatment (Marchese and Benovic, 2001; Shenoy et al., 2001; Jacob et al., 2005). In addition, wild-type β_1 -AR was reported not to undergo agonist-induced ubiquitination or down-regulation (Liang and Fishman, 2004). In contrast, Tanowitz et al. (2002) reported that mutation of all intracellular Lys residues to Arg in the δ opioid receptor did not affect agonist-induced endocytosis and proteolytic degradation of the receptor and its colocalization with lysosomes, indicating ubiquitination-independent down-regulation. However, Vps4/SKD-1 and Hrs are still required for sorting of the mutant δ opioid receptor from endosomes to multivesicular bodies to lysosomes (Hislop et al., 2004). Thus, requirement of ubiquitination for down-regulation seems to be receptor-dependent. There are two possibilities for the sorting of unubiquitinated hKOR for degradation. The result that pretreatment with the lysosome inhibitor chloroquine partially blocked Dyn A-induced hKOR-10KR down-regulation (supplemental data 2) indicates that some of the hKOR-10 KR is trafficked to lysosomes for degradation. In addition, there may be other factors involved in the trafficking of the hKOR. G protein-coupled receptor-associated sorting protein has been shown to interact with the C-terminal domains of the δ and κ opioid receptors (Whistler et al., 2002; Simonin et al., 2004) and participate in sorting the δ opioid receptor to lysosomes for degradation (Whistler et al., 2002). These results further support the notion that ubiquitination of hKOR regulates, but is not required for, its down-regulation.

Ubiquitinated hKOR Contains Predominantly Lys63-Linked Polyubiquitin Chains. There are seven Lys residues in ubiquitin: Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. Polyubiquitin chains can be linked by different Lys residues in ubiquitin; however, Lys48- and Lys63-linked polyubiquitin chains are the predominant ones. It has been recognized that Lys48-linked polyubiquitination targets proteins to proteasomes for degradation, whereas Lys63-linked polyubiquitination plays important roles in interactions with protein machineries involved in endocytic trafficking, inflammatory response, protein translation, and DNA repair (for review, see Pickart and Fushman, 2004).

We found that the K63R ubiquitin mutant greatly decreased Dyn A-promoted hKOR ubiquitination and down-regulation, but the K48R mutant did not (Fig. 7). These results indicate that agonist-promoted polyubiquitination of the hKOR occurs predominantly as Lys63-linked polyubiquitin chains, which is involved in its agonist-induced down-regulation. This is the first report that Lys63-linked polyubiquitination of a 7TMR is involved in its agonist-promoted down-regulation. Lys63-linked polyubiquitination has been shown to be involved in endocytic trafficking and degradation of epidermal growth factor and Trk A receptors (Geetha et al., 2005; Huang et al., 2006a) and dopamine transporter (Miranda et al., 2005).

Ubiquitination is a reversible process. Deubiquitinating enzymes remove ubiquitins from ubiquitinated proteins, but do not degrade ubiquitins, and, thus, allow ubiquitin to be re-used. Expression of HN-CYLD, a DN mutant of the deubiquitination enzyme CYLD, which breaks Lys63-linked but not Lys48-linked polyubiquitin chains, increased Dyn A-induced hKOR ubiquitination and down-regulation (Fig. 8). These results further support the role of Lys63-linked polyubiquitination of hKOR in its down-regulation. To the best of

our knowledge, this is the first report to show that deubiquitinating enzymes play a role in 7TMR down-regulation. The role of deubiquitinating enzymes in EGFR trafficking has been studied, yielding inconsistent results. Mizuno et al. (2005) reported that UBPY deubiquitinated the EGFR in endosomes and reduced EGFR degradation; however, Row et al. (2006) and Alwan and van Leeuwen (2007) showed that deubiquitination of EGFR by UBPY is required for EGFR degradation.

K63R ubiquitin greatly reduced Dyn A-promoted hKOR ubiquitination but modestly attenuated hKOR down-regulation. These observation is in accord with the notion that ubiquitination regulates, but is not required for, hKOR down-regulation. In addition, HN-CYLD dramatically enhanced Dyn A-promoted hKOR ubiquitination but increased hKOR down-regulation to a much lower extent. These results suggest that the level of ubiquitination without HN-CYLD expression may be sufficient to drive down-regulation, and higher ubiquitination levels do not increase down-regulation proportionally to the increase in ubiquitination. In addition, the immunoprecipitation procedure we used did not remove proteins associated with the receptor. Therefore, the possibility that HN-CYLD expression enhanced ubiquitination of associated proteins cannot be excluded.

Agonist-Promoted Ubiquitination of the hKOR Was Regulated by Receptor Phosphorylation, but Not By Receptor Internalization. We showed previously that GRK2-K220R and β -arrestin (319–418) reduced agonist-induced internalization of the hKOR (Li et al., 1999). In addition, GRK2-K220R attenuated, and S358N mutation in the hKOR eliminated, agonist-induced phosphorylation of hKOR (Li et al., 2002a). β -Arrestin (319–418) binds well to clathrin but not at all to receptor (Krupnick et al., 1997). In this study, we found that Dyn A did not stimulate ubiquitination of the hKOR-S358N mutant, and the dominant-negative mutants GRK2-K220R and β -arrestin (319–418) reduced Dyn A-stimulated hKOR ubiquitination. These results indicate that ubiquitination of the hKOR is enhanced by receptor phosphorylation and association of phosphorylated receptor with clathrin via β -arrestin. Similar observations were reported for the β_2 -AR (Shenoy et al., 2001) and the yeast α -factor receptor Ste2 (Hicke et al., 1998). Because dynamin I-K44A, an effective inhibitor of hKOR internalization (Li et al., 1999), did not affect its ubiquitination, agonist-stimulated ubiquitination of the hKOR does not require receptor internalization and hence occurs to the hKOR in the plasma membrane. β -Arrestin was found to be required for ubiquitination of V2 vasopressin receptor (Martin et al., 2003). How β -arrestin plays a role in the ubiquitination of hKOR needs further investigation. It is reasonable to speculate that agonist-promoted hKOR phosphorylation at Ser358 triggers events ultimately leading to ubiquitination at one or more of Lys residues at 338, 349, and 378 in the hKOR C-terminal domain.

Agonist-Dependent and -Independent 7TMR Ubiquitination. There are two pools of 7TMRs that are ubiquitinated: misfolded newly synthesized receptors and receptors on plasma membranes. The former is agonist-independent and the latter is agonist-dependent.

Because Dyn A is a hydrophilic ligand that activates only cell surface receptors, agonist-induced hKOR ubiquitination occurs to receptors on plasma membranes. Agonist treatment

has been found to enhance ubiquitination of several other 7TMRs, including the yeast α -factor (Hicke and Riezman, 1996; Roth and Davis, 1996), β_2 -AR (Shenoy et al., 2001), V2 vasopressin (Martin et al., 2003), PAR2 (Jacob et al., 2005), CXCR4 (Marchese and Benovic, 2001), and NK-1 (Cottrell et al., 2006) receptors, which is involved in endocytosis, trafficking of the receptors from endosomes to lysosomes.

There was a low level of basal ubiquitination of the hKOR, which was most likely the result of ubiquitination of newly synthesized misfolded receptors for endoplasmic reticulum-related degradation. Agonist-independent ubiquitination has been demonstrated for the δ opioid (Petaja-Repo et al., 2001), thyrotropin-releasing hormone (Cook et al., 2003), and calcium-sensing (Huang et al., 2006b) and platelet-activating factor (Dupré et al., 2003) receptors.

10KR mutation did not totally eliminate ubiquitination, perhaps because there may be ubiquitinated proteins immunoprecipitated with the hKOR. For example, Shenoy et al. (2001) demonstrated that after activation of the β_2 -adrenergic receptor, β -arrestin was ubiquitinated, which was important for internalization of the receptor.

Methods Used for Determination of hKOR Down-Regulation. We used both flow cytometry and immunoblotting to determine receptor down-regulation. Flow cytometry was used to quantify loss of cell surface receptor, whereas immunoblotting followed by quantitation detected reduction in the mature (55-kDa) form of the hKOR. In our previous study (Chen et al., 2006b) and in the current study, we found that two methods yielded similar levels of agonist-induced hKOR down-regulation. Radioligand binding was not used to determine down-regulation because it was difficult to remove Dyn A, and residual Dyn A interfered with binding.

Conclusion

Agonists promoted ubiquitination of the hKOR, which occurs after receptor phosphorylation but before internalization. The hKOR was ubiquitinated at the three Lys residues in the C-terminal domain, predominantly by Lys63-linked polyubiquitin chains. Agonist-promoted ubiquitination plays a regulatory, but not obligatory, role for agonist-induced down-regulation.

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