N-Glycosylation of the Human κ Opioid Receptor Enhances Its Stability but Slows Its Trafficking along the Biosynthesis Pathway†

Jian-Guo Li, Chongguang Chen, and Lee-Yuan Liu-Chen*

Department of Pharmacology and Center for Substance Abuse Research, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received March 5, 2007; Revised Manuscript Received June 12, 2007

ABSTRACT: We examined glycosylation of FLAG-hKOR expressed in CHO cells and determined its functional significance. FLAG-hKOR was resolved as a broad and diffuse 55-kDa band and a less diffuse 45-kDa band by immunoblotting, indicating that the receptor is glycosylated. Endoglycosidase H cleaved the 45-kDa band to ∼38 kDa but did not change the 55-kDa band, demonstrating that the 45-kDa band is *N*-glycosylated with high-mannose or hybrid-type glycan. Peptide−*N*-glycosidase F digestion of solubilized hKOR or incubation of cells with tunicamycin resulted in two species of 43 and 38 kDa, suggesting that the 43-kDa band is *O*-glycosylated. FLAG-hKOR was reduced to lower M_r bands by neuraminidase and *O*-glycosidase, indicating that the hKOR contains *O*-linked glycan. Mutation of Asn25 or Asn39 to Gln in the N-terminal domain reduced the M_r by ∼5 kDa, indicating that both residues were glycosylated. The double mutant hKOR-N25/39Q was resolved as a 43-kDa (mature form) and a 38-kDa (intermediate form) band. When transiently expressed, hKOR-N25/39Q had a lower expression level than the wild type. In CHO cells stably expressing the hKOR-N25/39Q, pulse−chase experiments revealed that the turnover rate constants (k_e) of the intermediate and mature forms were ∼3 times those of the wild type. In addition, the maturation rate constant (k_a) of the 43-kDa form of hKOR-N25/39Q was 6 times that of the mature form of the wild type. The hKOR-N25/39Q mutant showed increased agonist-induced receptor phosphorylation, desensitization, internalization, and downregulation, without changing ligand binding affinity or receptor−G protein coupling. Thus, *N*-glycosylation of the hKOR plays important roles in stability and trafficking along the biosynthesis pathway of the receptor protein as well as agonist-induced receptor regulation.

Opioid receptors were classified into at least three types, μ, δ, and κ, based on pharmacological and anatomical analyses (for reviews, see refs *1* and *2*). Activation of κ opioid receptors in vivo produces many effects including antinociception (*3*, *4*), psychotomimesis (*4*, *5*) and water diuresis (*3*, *4*). At the cellular level, κ opioid receptors are coupled through pertussis toxin-sensitive G proteins to affect a variety of effectors, which include adenylyl cyclase, potassium channels and calcium channels, and the p42/p44 mitogen-activated protein kinase pathway (for a review, see ref *6*). κ opioid receptors have been cloned from several species (for a review, see ref *7*) and belong to the rhodopsin subfamily of the seven transmembrane domain receptor (7TMR)[1] family.

Glycosylation is an important modification during maturation processes of many membrane-bound proteins. Initial *N*-linked glycosylation with high-mannose glycans occurs cotranslationally in the endoplasmic reticulum (ER). Some glucose and mannose molecules were trimmed from high-mannose glycans in the ER and Golgi apparatus. Addition of *N*-acetylglucosamine, galactose, and sialic acid occurs in the Golgi apparatus to become complex-type glycans. For some proteins, *O*-glycosylation occurs at Thr and Ser residues in the Golgi (*8*). Fully glycosylated proteins are transported to plasma membranes.

Many 7TMRs have been shown to be glycosylated, which is involved in cell surface expression and agonist-induced regulation of the receptors (*9*−*12*). Removing *N*-linked glycosylation by tunicamycin treatment or site-directed mutagenesis decreased expression on the cell surface of most 7TMRs studied, for example, the δ opioid (*13*) and calcitonin receptor-like receptors (*14*) and proteinase-activated receptor-2 (*15*), but did not affect others, such as the P(2)Y(12) (*16*) and metabotropic glutamate 1α (*17*) receptors. However, mechanisms underlying the role of *N*-glycosylation in cell surface expression of 7TMRs have rarely been elucidated.

In this study, we investigated whether the hKOR expressed in CHO cells contains *N*-linked and *O*-linked glycans and examined effects of *N*-glycosylation of hKOR on receptor properties, regulation, and trafficking. We found that *N*-

† This work was supported by National Institutes of Health Grants DA17302 and DA04745.

* Correspondence should be addressed to this author. Tel: 215-707-4188. Fax: 215-707-7068. E-mail: lliuche@temple.edu.

[1] Abbreviations: CHO cells, Chinese hamster ovary cells; CHO-FLAG-hKOR, clonal CHO cell lines stably expressing FLAG-tagged human κ opioid receptor; DDM, *n*-dodecyl β-D-maltoside; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FLAG-hKOR, FLAG-tagged human κ opioid receptor; 7TMR, seven transmembrane domain receptor; hKOR, human κ opioid receptor; PMSF, phenylmethanesulfonyl fluoride; PNGase F, peptide−*N*-glycosidase F; rKOR, rat κ opioid receptor; U50,488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide.

10.1021/bi700443j CCC: $37.00
Published on Web 08/21/2007

glycosylation was important for stability and agonist-induced regulation of the receptor protein but slowed its trafficking from ER to Golgi.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Diprenorphine (58 Ci/mmol), L-[^{35}S]methionine/cysteine (~1175 Ci/mmol), [^{32}P]orthophosphate (8500 − 9100 Ci/mmol) and [^{35}S]GTPγS (~1250 Ci/mmol) were purchased from Perkin-Elmer Co. (Boston, MA). Dynorphin A was purchased from Phoenix Pharmaceuticals (Belmont, CA). U50,488H and naloxone were provided by the National Institute on Drug Abuse (Bethesda, MD). Purified rabbit anti-FLAG antibody (F7425), anti-FLAG M2 affinity gel, M1 mouse anti-FLAG antibody, peroxidase-conjugated goat anti-rabbit IgG, tunicamycin, soybean trypsin inhibitor, *n*-dodecyl β-D-maltoside (DDM), 1,10-phenanthroline, benzamidine, MG132, and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 was purchased from Molecular Probes (Eugene, OR). Endoglycosidase H (Endo H) from *Streptomyces plicatus* (EC 3.2.1.96), neuraminidase from *Vibrio cholerae* (EC 3.2.1.18), and *O*-glycosidase from *Diplococcus pneumoniae* (EC 3.2.1.97) were obtained from Roche Applied Science (Indianapolis, IN). Peptide−*N*-glycosidase F (PNGase F) from *Flavobacterium meningosepticumi* (EC 3.5.1.52) was purchased from New England Biolabs (Beverly, MA). Pansorbin cells were from Calbiochem (San Diego, CA), and the SuperSignal West Pico chemiluminescent substrate kit was from Pierce Chemical Co. (Rockford, IL). Protease inhibitor cocktail tablets were purchased from Roche (Nutley, NJ).

Generation of FLAG-hKOR-N25Q, FLAG-hKOR-N39Q and FLAG-hKOR-N25/39Q cDNAs. cDNA encoding human κ opioid receptor with the N-terminal FLAG epitope (FLAG-hKOR) in pcDNA3 was generated previously (*18*). Using point mutations and the overlap polymerase chain reaction (PCR) method (*19*) with the FLAG-hKOR in pcDNA3 as the template, asparagine (Asn) at positions 25 and/or 39 in the N-terminal domain of FLAG-hKOR was replaced with glutamine (Gln) to generate two single mutants, FLAG-hKOR-N25Q and FLAG-hKOR-N39Q, and one double mutant, FLAG-hKOR-N25/39Q. Three mutated cDNAs were 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-N25/39Q in CHO Cells. Clonal CHO cells stably transfected with the FLAG-hKOR-N25/39Q cDNA (CHO-FLAG-hKOR-N25/39Q) were established, and a cell line with a similar receptor expression level to CHO cells stably transfected with the FLAG-hKOR cDNA (CHO-FLAG-hKOR) (*20*) was selected for this study. CHO-FLAG-hKOR and CHO-FLAG-hKOR-N25/39Q cells were cultured in Dulbecco's modified Eagle's medium F12 HAM supplemented with 10% fetal calf serum, 0.1 mg/mL geneticin, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C.

Western Blot. Western blot was performed to examine the expression of FLAG-hKOR proteins as described previously (*20*). Cells were solubilized with 2× Laemmli sample buffer (4% SDS, 0.1 M DTT, 20% glycerol, 62.5 mM Tris-HCl, pH 6.8), subjected to Tricine−SDS−PAGE, and transferred onto PDVF Immobilon membranes. Membranes were treated with blocking solution, incubated with the polyclonal FLAG antibody or rabbit antiserum against rKOR (371−380) and then 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 LAS1000 plus system and quantitated using the ImageGauge software (version 4.1; Fuji Photo Film Co. Ltd.).

Deglycosylation of Solubilized and Immunoprecipitated FLAG-hKOR. The assays were performed according to published procedures (*21*). In some experiments, cells were treated with 5 μg/mL tunicamycin at 37 °C for the indicated periods of time. Membranes of CHO-FLAG-hKOR cells were prepared as described previously (*22*). Membranes were washed with buffer A [50 mM sodium phosphate, pH 5.5, 50 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM 1,10-phenanthroline, 5 μg/mL leupeptin, 5 μg/mL STI, 10 μg/mL benzamidine] for Endo H or buffer B (same as buffer A, pH 7.5) for PNGase F. Then membranes were solubilized in the respective buffers containing 0.5% DDM (w/v) at 4 °C for 1 h. After 100000*g* centrifugation at 4 °C for 1 h, the supernatant was collected for deglycosylation of solubilized receptor or immunoprecipitation followed by deglycosylation. For deglycosylation of solubilized receptors, the supernatant was treated with 100 milliunits/mL Endo H or 200 kilounits/mL PNGase F at 37 °C for 16 h, and the reaction was stopped by adding SDS sample buffer. Alternatively, the supernatant was incubated overnight at 4 °C with M2 anti-FLAG antibody−agarose to immunoprecipitate FLAG-hKOR, and M2 anti-FLAG antibody resin was eluted with 1% (w/v) SDS and 50 mM sodium phosphate, pH 7.5. The eluate was diluted 10-fold with 0.5% (w/v) DDM in buffer A containing 1% 2-mercaptoethanol (Endo H), buffer B containing 1% 2-mercaptoethanol (PNGase F), or 50 mM sodium phosphate, pH 6.0, 0.5 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 10 μg/mL benzamidine (neuraminidase and *O*-glycosidase). The enzymes were added at final concentrations of 100 milliunits/mL (Endo H), 200 kilounits/mL (PNGase F), and 50 milliunits/mL (neuraminidase and *O*-glycosidase) and incubated at 37 °C for 16 h. Samples were resolved by SDS−PAGE and detected by immunoblotting.

[^{35}S]Met/Cys Labeling of hKOR and hKOR-N25/39Q and Kinetic Analysis of the Newly Synthesized Receptor. Experiments were performed using our modified procedure (*23*) of that described by Petaja-Repo et al. (*24*). For conducting [^{35}S]Met/Cys labeling, 1.2×10^6 cells were cultured into a 60 mm Petri dish, grown in complete medium for 24 h, preincubated with 2 mL of Met/Cys-free DMEM depletion medium at 37 °C for 1.5 h, and pulse-labeled with 150 μCi/mL L-[^{35}S]methionine/cysteine in fresh depletion medium. After 30 min labeling at 37 °C, the pulse phase was terminated by washing the cells one time with the chase medium (complete medium supplemented with 5 mM L-methionine) and then incubated with the chase medium for specified time periods. Following the chase phase, cells were detached using Versene buffer, washed once with PBS, pelleted by centrifugation at 2500*g* at 4 °C for 5 min, and stored at −80 °C until further studies.

Protein solubilization was accomplished by thawing the cells in 400 μL of TTSEC buffer (2% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail tablet, pH 7.4) with 1 h shaking at 4 °C. The supernatant was obtained by centrifugation at 13500*g* for 15 min. For better signal/noise ratio, antibody−Pansorbin precipitation was performed twice in tandem to purify the ^{35}S-labeled FLAG-hKOR. First, 400 μL of supernatant was incubated with 2 μg of polyclonal anti-FLAG antibody at 4 °C for 1 h and mixed with 20 μL of Pansorbin at 4 °C for an additional 1 h. The receptors adsorbed on Pansorbin cells were washed three times by repeated centrifugation and resuspension in TBS-T buffer and then eluted by incubating the pellets in 20 μL of 2× Laemmli sample buffer at room temperature for 15 min. Then 20 μL of supernatant was added into 380 μL of TTSEC buffer for the second round of the precipitation experiment. Adsorbed receptors were eluted with 50 μL of 2× Laemmli sample buffer for Tricine−SDS−PAGE on 8% separating gel. The gel was dried using a gel dryer (Bio-Rad Laboratories, Hercules, CA) and then exposed to a prebleached storage phosphor screen for 2 days. The autoradiogram was captured by a Cyclone Storage Phosphor System (Perkin-Elmer Life and Analytical Sciences). The intensities of radiolabeled receptor bands were quantitated with the OptiQuant software. The background signal of each lane was subtracted before performing quantitative data analysis.

Our previous data showed that CHO-FLAG-hKOR cells migrated as two bands of M_r 55 kDa (mature receptor) and 45 kDa (immature receptor) (*25*). Transformation of the immature receptor form to the mature form and turnover of both receptor forms appear to follow the first-order kinetics. All analyses were performed using Prism 3.0 to fit the data to the equations (*26*):

$$X = A(e^{-k_e t} - e^{-k_a t}) \qquad \text{(for mature form)}$$

$$X = Ae^{-k_e t} \qquad \text{(for immature form)}$$

where X is the amount of FLAG-hKOR, A is a constant for each equation, k_a is the transformation rate constant of the immature to mature receptor, k_e is the turnover rate constant of the mature or immature receptor, and t is the time of the chase. The turnover rate constant (k_e) means the fraction of the receptor degraded per unit of time. Half-life ($t_{1/2}$) is the time for the receptor to reduce by 50%, and it is equal to $0.693/k_e$.

Quantitation of Receptor Internalization and Surface Receptor by Fluorescence Flow Cytometry. Receptor internalization and surface receptor were determined by fluorescence flow cytometry assay according to our published procedure (*27*). Briefly, CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells in 12-well plates were treated with or without 10 μM U50,488H for 30 min (for internalization) or 1 μM dynorphin A for 4 h (for downregulation) at 37 °C. Cells were washed three times with ice-cold PBS buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl) and lifted with PBS buffer containing 0.5 mM EDTA. Cells were incubated with M1 anti-FLAG antibody (1 μg/mL) in 300 μL of Opti-MEMI reduced serum medium containing 1 mM $CaCl_2$ 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 μg/mL) in 300 μL of Opti-MEMI reduced serum medium containing 1 mM $CaCl_2$ for 45 min at 4 °C. Cells were washed three times with ice-cold PBS buffer containing 1 mM $CaCl_2$ and then resuspended with 300 μL of PBS buffer. Immunoreactivity of cell surface receptors was quantitated by fluorescence flow cytometry (FACScan; BD Biosciences, San Jose, CA).

κ Opioid Receptor Binding. Membrane preparations and receptor binding of CHO-FLAG-hKOR and CHO-FLAG-hKOR-N25/39Q cells were conducted with [^{3}H]diprenorphine in 50 mM Tris-HCl buffer as we described previously (*28*). Naloxone (10 μM) was used to define nonspecific binding. Saturation experiments were performed with various concentrations of [^{3}H]diprenorphine (ranging from 0.02 to 2 nM) to determine the total receptor number. Competitive inhibition of [^{3}H]diprenorphine binding was performed with [^{3}H]diprenorphine at a concentration of ∼0.3 nM and various concentrations of agonist U50,488H 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 about 15 μg of membrane protein. Bound and free ligands 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 Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Intact Cell Binding and Intracellular Receptors. hKOR binding on intact cells was conducted with [^{3}H]diprenorphine in Krebs−Ringer HEPES buffer solution as described previously (*28*). Briefly, CHO cells were transiently transfected with 10 μg of FLAG-hKOR cDNA or FLAG-hKOR-N25/39Q cDNA. After 48 h, cells were collected, and saturation binding of [^{3}H]diprenorphine to the intact cells was performed in Kreb's buffer solution (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.4). B_{max} and K_d values of [^{3}H]diprenorphine binding to intact cells were determined as mentioned above.

For assessment of intracellular receptors, binding to total and cell surface receptors was conducted. Binding was conducted with 2 nM [^{3}H]diprenorphine. Nonspecific binding for total and cell surface receptors was defined by binding in the presence of 10 μM naloxone and 1 μM dynorphin A, respectively. Naloxone, a hydrophobic ligand, binds to both cell surface and intracellular receptors, whereas dynorphin A, a hydrophilic ligand, binds only to cell surface receptors. Thus, the difference between total and cell surface receptor binding represents binding to the intracellular receptor pool (*28*).

[^{35}S]GTPγS Binding and U50,488H-Induced Desensitization. Membrane preparations and [^{35}S]GTPγS binding were performed according to Li et al. (*20*). Briefly, CHO-FLAG-hKOR and CHO-FLAG-hKOR-N25/39Q cells were treated with or without 1 μM U50,488H for 30 min and washed three times with 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 PMSF, 10 μM leupeptin, 10 mM sodium fluoride, and 10 mM tetrasodium pyrophosphate, pH 7.4) was added. The cell suspension was passed through a 1 mL 29_G3/8 syringe needle five times and centrifuged. Pellets were resuspended 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 of Smith et al. (*29*), and membranes were frozen at −80 °C until use. About 10 μg of membrane protein was incubated with or without different concentrations of U50,488H, 15 μM GDP, and 0.2 nM [^{35}S]GTPγS in the reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 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 [^{35}S]GTPγS were separated by filtration with GF/B filters. Radioactivity on filters was determined by liquid scintillation counting.

Phosphorylation of the κ Opioid Receptors. Phosphorylation was conducted according to our published procedure (*27*) with some modifications. CHO-FLAG-hKOR and CHO-FLAG-hKOR-N25/39Q cells were transferred into 60 mm dishes and cultured overnight to confluence. Cells were then grown in 2 mL per well of phosphate-free medium at 37 °C for 2 h. [^{32}P]Orthophosphate (0.25 mCi/well) was added and incubated for another 2 h, and medium was aspirated. Cells were incubated with 1 μM (−)U50,488H for 5 and 20 min at 37 °C, cooled on ice, and washed three times with ice-cold PBS. All subsequent steps were carried out at 4 °C. Cells were solubilized for 1 h with solubilization buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 20 nM calyculin A, and 10% complete protease inhibitor cocktail, pH 7.5) and centrifuged at 13000*g* for 20 min. Immunoprecipitation of the receptor was performed with rabbit anti-FLAG polyclonal antibody followed by Pansorbin (final 1/200, 4 °C, 1 h). The mixture was washed three times by repeated centrifugation and resuspension in TBS-T buffer and then eluted by incubating the pellets in 50 μL of 2× Laemmli sample buffer. Then 50 μL of supernatant was added into 900 μL of TTSEC buffer and 50 μL anti-FLAG M2 affinity gel for the precipitation experiment again. Immunoprecipitated materials were dissolved in 2× Lammeli sample buffer and subjected to 8% SDS−polyacrylamide gel electrophoresis, and ^{32}P was detected by use of a phosphoimager (Cyclone; Packard BioScience Co.). Quantitation of receptor phosphorylation was performed with the OptiQuant software program.

Statistical Analysis. For comparison of multiple groups, data were analyzed with analysis of variance to determine if there were significant differences among groups using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). 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

Identification of FLAG-hKOR Expressed in CHO Cells by Immunoblotting. When CHO-FLAG-hKOR cell extracts were resolved with SDS−PAGE and the receptor was detected with immunoblotting with anti-FLAG polyclonal antibody, FLAG-hKOR was revealed as a broad and diffuse 55-kDa band and a less diffuse 45-kDa band (Figure 1A). In untransfected CHO cells, no protein bands were detected (Figure 1A), indicating that the two bands represent FLAG-hKOR. The two bands were also detected by use of a rabbit antiserum against a synthetic peptide corresponding to amino acids 371−380 in the C-terminal domain of the rat KOR (data not shown). The broad and diffuse nature of the bands indicates that the receptor is glycosylated. These results are similar to what we reported previously (*25*).

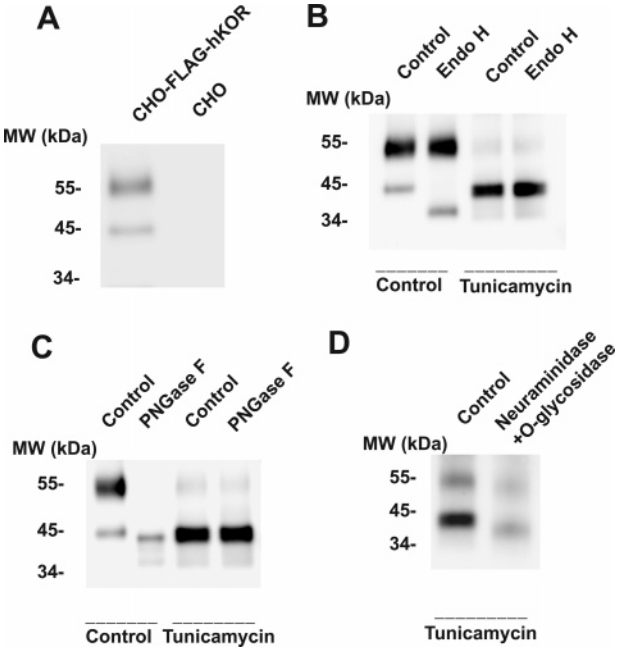

FIGURE 1: Deglycosylation of FLAG-hKOR with Endo H, PNGase F, and *O*-glycosidase. (A) Immunoblotting of FLAG-hKOR stably expressed in CHO cells. CHO-FLAG-hKOR cells or untransfected CHO cells were lysed and subjected to SDS−PAGE. Immunoblotting was performed using rabbit anti-FLAG antibody as described in Experimental Procedures. (B−D) Deglycosylation of FLAG-hKOR with Endo H, PNGase F, and *O*-glycosidase. CHO-FLAG-hKOR cells were pretreated with or without 5 μg/mL tunicamycin at 37 °C for 40 h and solubilized, and FLAG-hKOR was immunoprecipitated with M2 anti-FLAG antibody conjugated agarose and incubated for 16 h at 37 °C with or without (B) 100 milliunits/mL Endo H, (C) 200 kilounits/mL PNGase F, and (D) 50 milliunits/mL neuraminidase and 50 milliunits/mL *O*-glycosidase. Reactions were stopped by adding 2× Laemmli sample buffer, followed by SDS−PAGE and immunoblotting using the rabbit anti-FLAG antibody as described in Experimental Procedures. Each figure represents one of three independent experiments performed with similar results.

Endo H Digested the 45-kDa, but Not the 55-kDa, Species. Endo H selectively removes *N*-linked glycans of high mannose and/or hybrid types from glycoproteins but not complex type (*30*). Treatment of solubilized receptors with Endo H reduced the 45-kDa band to about 38 kDa but did not change the 55-kDa species (Figure 1B), indicating the 45-kDa protein contains unprocessed high mannose and/or hybrid types of *N*-linked glycans. By pulse−chase experiments, we have demonstrated that the 45-kDa form is a precursor for the 55-kDa species (*25*). The resistance of the 55-kDa band to Endo H treatment (Figure 1B) suggests that this receptor species contains complex type *N*-linked glycans.

PNGase F Treatment Yielded Two Bands of 43 and 38 kDa. PNGase F removes all types of *N*-linked glycans from glycoproteins (*30*). Treatment of solubilized FLAG-hKOR with 200 kilounits/mL PNGase F yielded two bands of M_r 43 and 38 kDa (Figure 1C). These findings, along with the Endo H results, indicate that the 55-kDa protein contains complex type *N*-linked glycans. Integral membrane proteins containing complex type *N*-linked glycans are located in

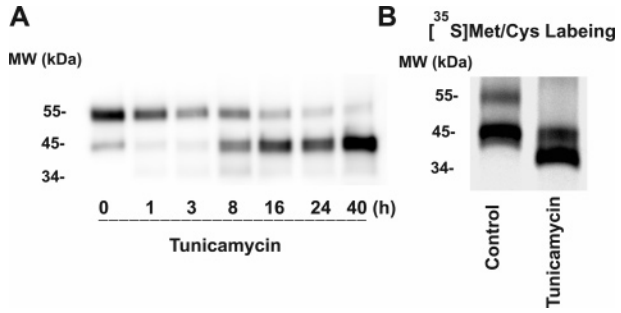

FIGURE 2: Effects of tunicamycin treatment of cells on FLAG-hKOR. (A) Time course of tunicamycin treatment. CHO-FLAG-hKOR cells were treated with 5 μg/mL tunicamycin at 37 °C for different time periods and solubilized with 2× Laemmli sample buffer, followed by SDS−PAGE and immunoblotting using rabbit anti-FLAG antibody as described in Experimental Procedures. (B) Effects on newly synthesized FLAG-hKOR. CHO-FLAG-hKOR cells were pretreated with 5 μg/mL tunicamycin for 3 h, labeled with [^{35}S]methionine/cysteine for the last 1 h, and solubilized. Solubilized materials were incubated with M2 anti-FLAG antibody conjugated agarose for immunoprecipitation of FLAG-hKOR, and the immunoprecipitation procedure was repeated as described in Experimental Procedures. Immunoprecipitated materials were resolved by SDS−PAGE, and autoradiography was performed. Each figure represents one of three independent experiments performed with similar results.

plasma membranes or in trans Golgi. In addition, since not all protein bands were reduced to 38 kDa, the 43-kDa species may contain additional modifications, most likely *O*-glycosylation.

Effect of Tunicamycin Treatment. Tunicamycin inhibits the synthesis of all *N*-linked glycoproteins by blocking the transfer of *N*-acetylglucosamine 1-phosphate to dolichol monophosphate (*30*). Treatment of cells with tunicamycin (5 μg/mL) reduced the amounts of the 55-kDa band gradually, and at 40 h, a residual amount still remained. Concomitantly, the 45-kDa band disappeared quickly, and at 3 h, only a faint band was left. In contrast, tunicamycin increased that of the 43-kDa species in a time-dependent manner (Figure 2A), and the 43-kDa species became the predominant species after 16 h (Figure 2A). In addition, at 8 h, a faint 38-kDa band appeared. The 43-kDa form was not digested by Endo H (Figure 1B) or PNGase F (Figure 1C), indicating that this form does not contain *N*-linked glycans.

FLAG-hKOR expression following tunicamycin treatment, as detected by immunoblotting at one time point, represents the combination of newly synthesized receptors and remaining existing receptors. For examination of effects of tunicamycin on newly synthesized receptors, CHO-FLAG-hKOR cells were incubated with [^{35}S]methionine/cysteine for 1 h to label the newly synthesized protein pool followed by immunoprecipitation of FLAG-hKOR. Autoradiography revealed FLAG-hKOR as two labeled bands of 55 and 45 kDa (Figure 2B), similar to those detected by immunoblotting. Treatment with tunicamycin resulted in FLAG-hKOR being synthesized as two species of 43 and 38 kDa (Figure 2B), identical to the bands produced by PNGase F treatment. These results further demonstrate that the 55- and 45-kDa species contain *N*-linked glycans.

O-Linked Glycosylation of the FLAG-hKOR. To investigate whether the FLAG-hKOR contains *O*-linked glycans, we subjected the 43-kDa species induced by tunicamycin treatment to deglycosylation using *O*-glycosidase and neuraminidase. *O*-Glycosidase removes unsubstituted *O*-linked galactose−*N*-acetylgalactosamine disaccharides from glycoproteins after the terminal sialic acids have been removed by neuraminidase (*31*). The 43-kDa species was reduced to 38−40-kDa bands by neuraminidase plus *O*-glycosidase (Figure 1D), indicating that the 43-kDa species contains *O*-linked glycans. In addition, the M_r of the residual 55-kDa band was reduced by ~4 kDa. *O*-Glycosidase alone did not cleave FLAG-hKOR (data not shown), indicating the presence of terminal sialic acid in *O*-linked glycans.

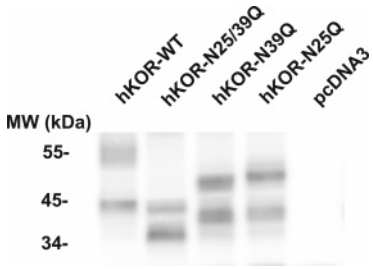

FIGURE 3: N25 and N39 are two *N*-linked glycosylation sites in the N-terminal domain of the hKOR. CHO cells were transiently transfected with FLAG-hKOR, FLAG-hKOR-N25Q, FLAG-hKOR-N39Q, FLAG-hKOR-N25/39Q, or the vector pcDNA3. Approximately 2 days later, cells were solubilized with 2× Laemmli sample buffer and subjected to SDS−PAGE. Immunoblotting was performed with polyclonal FLAG antibody as described in Experimental Procedures. Each figure represents one of three independent experiments performed with similar results.

Mutations of Asn25 and Asn39 to Gln in the N-Terminal Domain of FLAG-hKOR Abolished Glycosylation. There are two putative *N*-glycosylation sites (N-X-S/T, X represents any amino acid) in the N-terminal domain of the hKOR (Asn25 and Asn39). To determine whether these two sites are *N*-glycosylated, we generated two single mutants, FLAG-hKOR-N25Q and FLAG-hKOR-N39Q, and one double mutant, FLAG-hKOR-N25/39Q, by replacing Asn at positions 25 and/or 39 with Gln. Each of the mutants or the vector pcDNA3 was transiently transfected into CHO cells. Immunoblotting showed that M_r's of mature N25Q, N39Q, and N25/39Q mutants were 52, 50, and 43 kDa, respectively (Figure 3). In addition, those of the immature forms were 42, 42, and 38 kDa, respectively. These results indicate that both N25 and N39 are glycosylated. The observation that the intermediate form of the N25/39Q mutant had a M_r (38 kDa) identical to that produced by Endo H, PNGase F, and tunicamycin treatments indicates that Asn25 and Asn39 are the only N-glycosylation sites and that the core peptide of the hKOR has a M_r of 38 kDa.

Mutation of N-Glycosylation Sites of hKOR Decreased Receptor Expression. CHO cells were transiently transfected with 10 μg of FLAG-hKOR cDNA or FLAG-hKOR-N25/39Q cDNA. After 48 h, cells were collected, and saturation binding of [^{3}H]diprenorphine to the receptors was performed on intact cells. B_{max} and K_d values of [^{3}H]diprenorphine for FLAG-hKOR were determined to be 260.1 ± 2.7 fmol/10^6 cells and 0.38 ± 0.01 nM and for FLAG-hKOR-N25/39Q were 151.2 ± 1.6 fmol/10^6 cells and 0.33 ± 0.01 nM (Figure 4A). The results indicate that mutation of *N*-glycosylation sites of hKOR significantly decreases receptor expression ($P < 0.05$, by Student's *t* test) but does not affect the affinity.

Mutation of N-Glycosylation Sites of hKOR Did Not Change Receptor Binding Affinity for the Antagonist [^{3}H]-

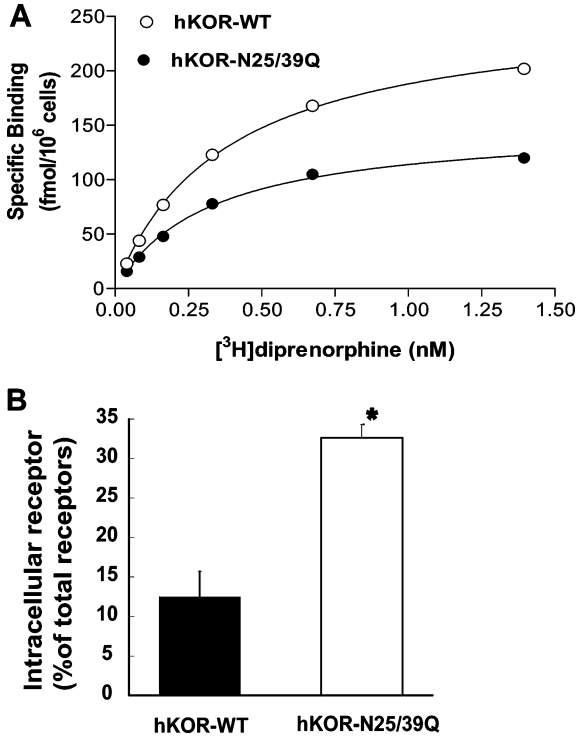

FIGURE 4: Elimination of *N*-glycosylation of hKOR decreased total receptor expression but enhanced the percent of receptor located intracellularly. (A) CHO cells were transiently transfected with FLAG-hKOR or FLAG-hKOR-N25/39Q. Approximately 2 days later, cells were collected, and [^{3}H]diprenorphine binding was performed on intact cells using 10 μM naloxone to define nonspecific binding as described in Experimental Procedures. Each value represents the mean ± SEM of three independent experiments. (B) Intracellular receptors were determined by binding of [^{3}H]-diprenorphine to intact CHO cells stably transfected with the receptors (CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells) in the presence of membrane-permeable ligand naloxone or nonpermeable ligand dynorphin A to define total and cell surface receptors, respectively. Intracellular receptors are expressed as the percent of total receptors. Each value represents the mean ± SEM of three independent experiments.

Diprenorphine and the Agonist U50,488H. CHO cells stably expressing FLAG-hKOR-N25/39Q were established, and one clonal cell line with a similar receptor expression level as CHO-FLAG-hKOR cells was selected for the subsequent experiments. Saturation binding of [^{3}H]diprenorphine and competitive inhibition of [^{3}H]diprenorphine binding by U50,-488H were performed on membranes prepared from both cell lines. The hKOR wild type and the hKOR-N25/39Q mutant had similar K_d values for [^{3}H]diprenorphine and comparable K_i values for U50,488H (Table 1). These results indicate that *N*-glycosylation of hKOR does not overtly change the receptor binding pocket.

The levels of total and cell surface receptors of hKOR and the N25/39Q mutant were determined by [^{3}H]diprenorphine binding to the intact CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cell in the presence of naloxone, a membrane-permeable ligand, and dynorphin A, a membrane-impermeable ligand, to define nonspecific binding, respectively. The difference between binding to total and cell surface receptor represents intracellular receptor binding. We found that 32.6 ± 1.7% of the total N25/39Q mutant and 12.5 ± 3.2% of the total wild-type receptor were present intracellularly ($P < 0.05$, by Student's *t* test) (Figure 4B).

Table 1: Receptor Expression Levels and Ligand Binding Affinities of the hKOR-WT and hKOR-N25/39Q Stably Expressed in CHO Cells[a]

	[^{3}H]diprenorphine		U50,488H
	B_{max} (pmol/mg of protein)	K_d (nM)	K_i (nM)
hKOR-WT	1.7 ± 0.2	0.12 ± 0.01	1.5 ± 0.1
hKOR-N25/39Q	2.4 ± 0.1	0.18 ± 0.01	1.8 ± 0.2

[a] Membranes of cells expressing the FLAG-hKOR or FLAG-hKOR-N25/39Q were prepared, and B_{max} and K_d values of [^{3}H]diprenorphine and the K_i value of U50,488H were determined as described in Experimental Procedures. Each value represents the mean ± SEM of three independent experiments.

Thus, the mutant has a higher level of intracellular receptors than the wild type. Even so, the majority of the mutant receptor is present on the cell surface.

Elimination of hKOR N-Glycosylation Did Not Affect Agonist-Induced [^{35}S]GTPγS Binding but Enhanced Receptor Desensitization. U50,488H-stimulated [^{35}S]GTPγS binding to membranes of cells expressing the wild type and the N25/39Q mutant was examined with or without pretreatment with U50,488H. Without U50,488H pretreatment, the EC_{50} and E_{max} values of U50,488H for both receptors were similar (Figure 5A), indicating that the *N*-glycosylation of hKOR does not affect receptor coupling to the G protein. Pretreatment with U50,488H induced desensitization of the N25/39Q receptor more profoundly than the wild type, reducing the E_{max} by about 55% and by 23%, respectively (Figure 5A). These results suggested that *N*-glycosylation of hKOR plays a role in agonist-induced receptor desensitization.

Elimination of hKOR N-Glycosylation Enhanced Agonist-Promoted Receptor Phosphorylation. Our previous study indicated that U50,488H (1 μM) induced maximal hKOR phosphorylation in 15 min (*20*). Both CHO-FLAG-hKOR and CHO-FLAG-hKOR-N25/39Q cells were treated with U50,488H for 5 and 20 min, and receptor phosphorylation was determined. The two time points were used for sub-maximal and plateau levels, respectively, and cell numbers were adjusted to have similar numbers of receptors. The hKOR-N25/39Q mutant showed higher levels of phosphorylation at both time points compared to the wild type (Figure 5B,C). Thus, elimination of hKOR *N*-glycosylation significantly enhances agonist-induced receptor phosphorylation.

Abrogation of N-Glycosylation of hKOR Enhanced Agonist-Induced Receptor Internalization and Downregulation. We examined whether N25/39Q mutations affected internalization and downregulation of the hKOR by FACS of cell surface receptors. Following incubation with 1 μM U50,-488H for 30 min, the N25/39Q mutant underwent a higher level of internalization than the wild type (Figure 6A) (74.1 ± 1.0% vs 42.4 ± 1.7%, $P < 0.05$, by Student's *t* test).

We reported recently that pretreatment with U50,488H for 4 h caused a lower extent of downregulation than with dynorphin A(1−17) (*23*) although the two agonists promoted similar levels of internalization (*27*). It was attributed to the finding that U50,488H, a membrane-permeable ligand, functioned as a pharmacological chaperone, but dynorphin A, a membrane-impermeable ligand, did not (*23*). Thus, we examined agonist-induced downregulation using dynorphin A instead of U50,488H.

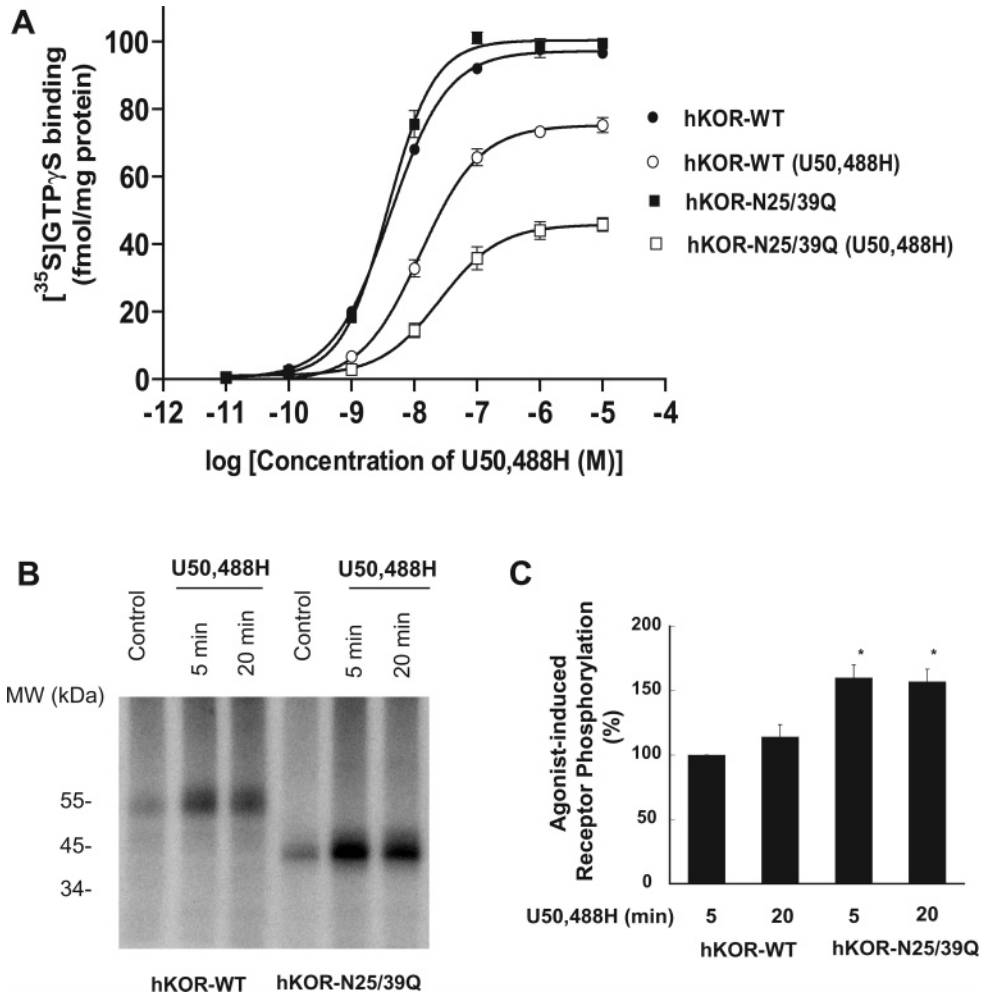

FIGURE 5: Abolition of *N*-glycosylation of hKOR enhanced agonist-induced receptor desensitization and phosphorylation without changing stimulation of [^{35}S]GTPγS binding. (A) CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells were incubated with or without 1 μM U50,488H for 30 min and washed. Membranes were prepared, and U50,488H-stimulated [^{35}S]GTPγS binding were determined as described in Experimental Procedures. (B) CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells were incubated with or without 1 μM U50,-488H for 5 and 20 min, and phosphorylation of the receptor was determined as described in Experimental Procedures. Cell numbers were adjusted so that similar receptor levels, determined by [^{3}H]diprenorphine binding, were present for the wild-type and mutant receptors. Each figure represents one of three independent experiments performed with similar results. (C) Quantitation of the results shown in (B) was performed using the OptiQuant software. Basal phosphorylation was subtracted from U50,488H-induced receptor phosphorylation, and each time point was normalized against U50,488H-induced phosphorylation of the wild-type hKOR at 5 min. Each value represents the mean ± SEM of three independent experiments. *, $P < 0.05$, compared with the hKOR wild type (5 min) by two-tailed Student's *t* test.

Dynorphin A (1 μM for 4 h) promoted a higher level of downregulation of the N25/39Q mutant than the wild type (88.8 ± 0.1% vs 67.1 ± 0.6%, $P < 0.05$, by Student's *t* test) (Figure 6B).

The levels of U50,488H-induced internalization and dynorphin A-promoted downregulation were similar to those reported previously (*23*, *27*). These results indicate that lack of *N*-glycosylation of hKOR makes it easier for the receptor to undergo agonist-induced internalization and downregulation.

Elimination of N-Glycosylation of hKOR Increased Rates of Generation and Degradation of the Mature Receptor (43-kDa Form) and the Degradation Rate of the Core Peptide (38-kDa Form). The pulse−chase technique was employed to determine generation and turnover rates of both forms of hKOR and hKOR-N25/39Q. As shown in Figure 7, after pulse labeling, most of the labeled FLAG-hKOR and FLAG-hKOR-N25/39Q existed as the 45- and 38-kDa forms, respectively. During the chase process, the 45-kDa spices of the FLAG-hKOR gradually decreased, and the 55-kDa mature receptor gradually increased. The 55-kDa form reached the highest level at 2 h (Figure 7A). For the hKOR-N25/39Q, the 38-kDa form was converted into the 43-kDa form, and the highest level of the latter was reached at 1 h (Figure 7A). Kinetic analyses of densitometry data showed that the transformation rate from the immature form (38 kDa) to the mature form (43 kDa) of hKOR-N25/39Q was increased by about 6-fold, compared with that from the 45- to 55-kDa form of the wild-type hKOR (Figure 7B and Table 2). Thus, lack of *N*-glycosylation enhances the maturation rate.

In addition, compared with those of the wild type, the turnover rates of both the immature and matue forms of hKOR-N25/39Q were significantly higher. The half-lives of these two forms of hKOR-N25/39Q were decreased by 3- and 2.7-fold, respectively, compared with those of the wild type (Figure 7C and Table 2). These results indicate that *N*-glycosylation of the hKOR enhances the stability of both forms of the receptor.

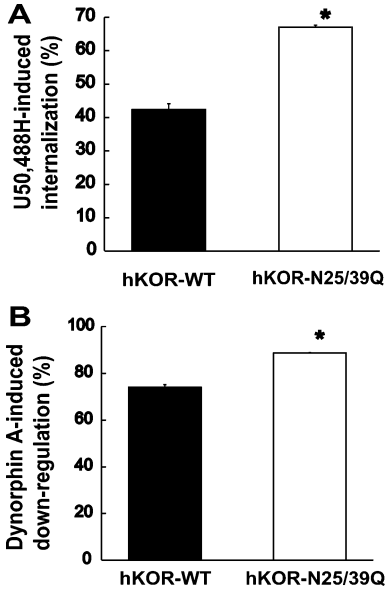

FIGURE 6: Elimination of *N*-glycosylation of hKOR enhanced agonist-induced receptor internalization and downregulation. CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells were treated with or without (A) 1 μM U50,488H for 30 min for internalization or (B) 1 μM dynorphin A for 4 h for downregulation. Receptor internalization or downregulation was determined by the fluorescence flow cytometry assay as described in Experimental Procedures. Each value represents the mean ± SEM of three independent experiments. *, $P < 0.05$, compared with the control group by two-tailed Student's *t* test.

DISCUSSION

The results herein indicate that the hKOR undergoes *N*- and *O*-linked glycosylation. Both N25 and N39 in the N-terminal domain are glycosylated and are the only *N*-glycosylation sites. Elimination of *N*-glycosylation enhances trafficking of the receptor from ER to Golgi but reduces the hKOR expression level, which may be attributed to the observed lower stability of the mutant receptor proteins. In addition, the hKOR mutant lacking *N*-glycosylation displays an increased intracellular receptor pool. Abolition of *N*-glycosylation enhances agonist-induced receptor phosphorylation, desensitization, internalization, and downregulation. To the best of our knowledge, this is the first report that *N*-glycosylation of a 7TMR *decreases* trafficking of the receptor along the biosynthesis pathway, in contrast to other 7TMRs, for which *N*-glycosylation increased trafficking from the ER to the cell surface (*33*). This is also the first definitive demonstration that *N*-glycosylation enhances stability of mature 7TMRs.

The hKOR Is N- and O-Glycosylated. N25Q or N39Q mutation in the N-terminal domain reduced the M_r of the hKOR, and the combined N25/39Q mutations had an additive effect, indicating that both N25 and N39 are glycosylated. Both PNGase and tunicamycin treatments, which remove all *N*-linked glycosylation, yielded two bands of 43 and 38 kDa. N25/39Q mutations yielded the same two bands, indicating that these are the only two sites that are *N*-glycosylated. Treatment with neuraminidase and *O*-glycosidase reduced the M_r of both the 55- and 43-kDa bands. Thus, the hKOR is *O*-glycosylated, and the 43-kDa band represents the *O*-glycosylated product of the 38-kDa band. This observation that the hKOR is *N*- and *O*-glycosylated is similar to the findings that the δ opioid receptor contains both *N*-linked and *O*-linked glycans (*13*, *21*). The μ opioid receptor was found to contain *N*-linked glycans (*32*); however, whether it is *O*-glycosylated has not been examined.

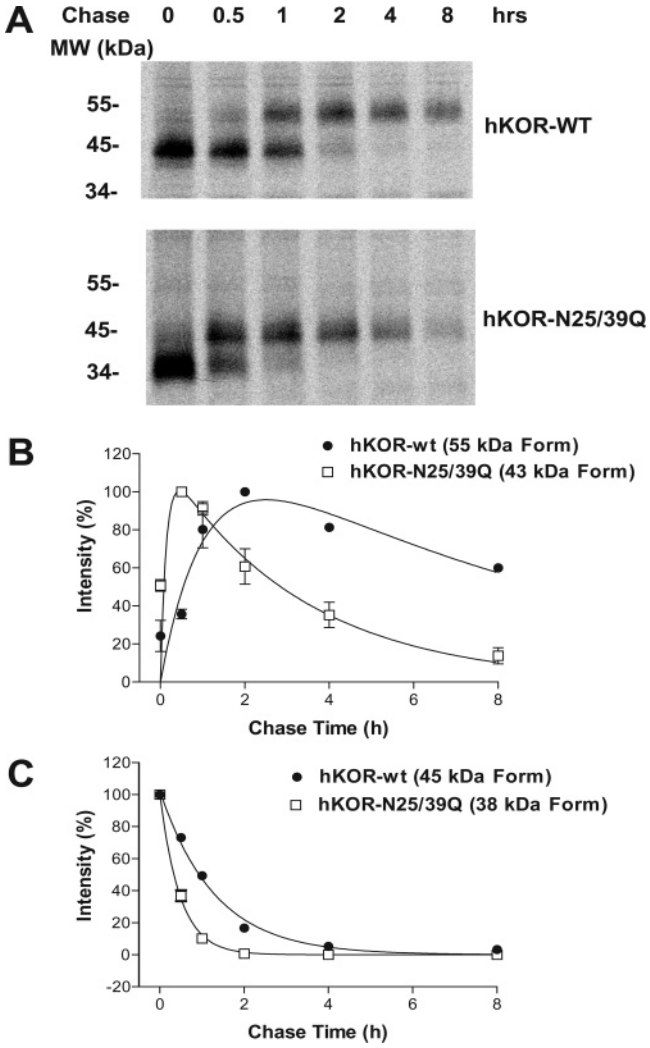

FIGURE 7: Effect of lack of *N*-glycosylation of hKOR on kinetics of newly synthesized hKOR and hKOR-10KR. CHO-FLAG-hKOR or CHO-FLAG-hKOR-N25/39Q cells were pulse-labeled with 150 μCi/mL of [^{35}S]Met/Cys at 37 °C for 30 min, then [^{35}S]Met/Cys was removed, and the cells were incubated with complete medium containing 5 mM methionine (chase) for the indicated time. After chase, cells were harvested and solubilized, and proteins were immunoprecipitated with anti-FLAG antibodies. Immunoprecipitated materials were resolved with SDS−PAGE followed by gel drying and autoradiography as described in Experimental Procedures. (A) Time courses of generation and turnover of the 55- and 45-kDa forms of the hKOR and 43- and 38-kDa forms of the hKOR-N25/39Q. The figure represents one of the three independent experiments performed with similar results. (B, C) Quantitation of the autoradiography results of (B) the 55-kDa form of the hKOR and the 43-kDa form of the hKOR-N25/39Q and (C) the 45-kDa form of the hKOR and the 38-kDa form of the hKOR-N25/39Q. Densitometric analysis was performed using the OptiQuant software. Each point represents the mean ± SEM of three independent experiments. Curve fitting of the data was performed using Prism 3.0 with equations shown in Experimental Procedures.

Glycosylation of the hKOR most likely occurs in a similar manner as that of other membrane-bound proteins. Initial *N*-linked glycosylation with high-mannose glycans occurs cotranslationally in the ER, followed by trimming of glucose and mannose in the ER and cis Golgi, and then addition of *N*-acetylglucosamine, galactose, and sialic acid in the Golgi

Table 2: Comparison of Kinetic Parameters of Both Newly Generated Receptor Species, the 55- and 45-kDa Forms of the hKOR and the 43- and 38-kDa Forms of the hKOR-N25/39Q[a]

		mature form WT 55 kDa N25/39Q 43 kDa	intermediate WT 45 kDa N25/39Q 38 kDa
generation rate constant (k_a, h^{-1})	hKOR-WT	0.99 ± 0.09	
	hKOR-N25/39Q	6.20 ± 0.05[b]	
turnover rate constant (k_e, h^{-1})	hKOR-WT	0.12 ± 0.001	0.78 ± 0.04
	hKOR-N25/39Q	0.34 ± 0.09[b]	2.12 ± 0.18[b]
half-life ($t_{1/2}$, h)	hKOR-WT	6.01 ± 0.04	0.99 ± 0.09
	hKOR-N25/39Q	2.25 ± 0.45[b]	0.34 ± 0.02[b]

[a] All parameters were determined from the data in Figure 7 as described in Experimental Procedures. The data shown in the table are the mean and SEM of three independent experiments. [b] $P < 0.05$, compared to the hKOR-WT group using two-tailed Student's *t*-test.

to form complex type glycans. Some high-mannose glycans are not trimmed back to the core and contain additional mannose residues. *O*-Glycosylation at Ser and Thr residues occurs in the Golgi.

The 45-kDa Form Is Located Intracellularly. We demonstrated previously that when cells were subjected to surface biotinylation, only the 55-kDa form of the hKOR was biotinylated (*23*). In addition, agonist-induced hKOR phosphorylation occurred only to the 55-kDa form (Figure 5B) (*20*). Moreover, after treatment with brefeldin A, which disrupts the Golgi apparatus and causes Golgi enzymes to be retrogradely transported to ER, there was a new hKOR form with a M_r of 51 kDa, which was generated from the 45-kDa species (*23*). These results indicate that the 45-kDa form of the hKOR resides in the ER and perhaps cis Golgi.

N-Glycosylation of hKOR and Receptor Biosynthesis. Mutation of the two *N*-glycosylation sites of hKOR decreased receptor expression when both the wild type and the mutant were transiently expressed. This result is consistent with observations on several 7TMRs, for example, the vasopression 1a receptor (*9*) and proteinase-activated receptor-2 (*15*). The lower expression level of the hKOR-N25/39Q is most likely attributed to the much faster degradation of the mature mutant receptor (Figure 7).

We found that the transformation rate from the intermediate form (38 kDa) to the mature form (43 kDa) of hKOR-N25/39Q was increased by about 6-fold, compared with that from the 45- to 55-kDa form of the wild type (Figure 7B and Table 2). Thus, removal of *N*-glycosylation of the hKOR increases receptor trafficking from the ER to the Golgi/cell surface. *N*-Glycosylation has been shown to be important for proper folding of newly synthesized proteins before exiting the ER. Elimination of *N*-glycosylation allows proteins to skip the steps involved in folding, which may accelerate their exit from the ER (*33*). This is the first report that abrogation of *N*-glycosylation enhances the receptor maturation process. Whether this is true for other 7TMRs remains to be determined.

We demonstrated that the $t_{1/2}$ of the intermediate (38 kDa) and mature (43 kDa) forms of the hKOR-N25/39Q were shortened by 3- and 2.7-fold, respectively, compared with those of the wild-type hKOR (Table 2). Thus, lack of *N*-glycosylation enhances agonist-independent degradation of the 43-kDa form of the hKOR-N25/39Q. Degradation of the 43-kDa band may occur on the cell surface, perhaps due to lack of extracellular binding of lectin to the *N*-linked glycan, which leads to degradation of the receptor or internalization and downregulation of the receptor, analogous to what happens to glucose transporter 2 (Glut-2) in pancreatic β cells (*34*). Glycosylation in the Golgi plays an important role in maintaining cell surface expression of Glut-2 in pancreatic β cells. Recent studies (*34*) have shown that decreased cell surface Glut-2 expression by genetic deletion of the Golgi glycosylation enzyme is due to a reduction in the cell surface Glut-2 half-life but not from a defect in trafficking from the Golgi apparatus to the cell surface. In addition, glycosylation of Glut-2 produces an *N*-glycan ligand for binding of multiple lectins in extracellular regions that reduces the rate of Glut-2 endocytosis (*34*).

The reduced half-life of the 38-kDa form of the hKOR-N25/39Q may be attributed to faster conversion to the 43-kDa form. In addition, the possibility cannot be excluded that lack of *N*-glycosylation may enhance ER-associated degradation of the 38-kDa form. These findings indicate that *N*-glycosylation of hKOR is important for stability of the receptor proteins.

Following tunicamycin treatment, the 38-kDa band was prominently present in the newly synthesized receptor pool labeled with [^{35}S]Met/Cys (Figure 2B) but appeared as a faint band with immunoblotting in the steady state of the hKOR in cells (Figure 2A). It is likely due to the short half-life of the 38-kDa band.

The 43-kDa Form of hKOR-N25/39Q Is Mostly on the Cell Surface. About 67% of the total N25/39Q receptor was on the cell surface, as determined by ligand binding. Thus, the 43-kDa receptor protein, which is devoid of *N*-glycosylation, but is *O*-glycosylated, was mostly located on the cell surface. This is consistent with the fact that *O*-glycosylation occurs in the trans Golgi, prior to protein being transported to the cell surface (*8*).

A Higher Percentage of Total hKOR-N25/39Q Is Intracellular. Lack of *N*-glycosylation resulted in higher levels of intracellular receptors as the percent of total receptors compared with the wild-type receptor (33% vs 13%, Figure 4B). This may be due to the greatly accelerated conversion of the intermediate (38 kDa) to the mature form of the N25/39 kDa, exceeding the capacity of the transport machinery. It may also be the result of less efficient insertion of the receptor into plasma membranes since *N*-glycosylation has been shown to be important for protein trafficking from the Golgi to the plasma membrane (*35*). In addition, we reported previously that the hKOR underwent constitutive internalization (*28*).

N-Glycosylation of hKOR and Receptor Binding Affinity and Signal Transduction. Our observations that *N*-glycosylation of hKOR did not affect its ligand binding affinity for [^{3}H]diprenorphine and U50,488H or agonist-induced G protein activation are consistent with observations on many 7TMRs. In NG108-15 cells, tunicamycin treatment did not alter the K_d value of [^{3}H]diprenorphine for δ opioid receptors (*13*). Nonglycosylated mutants of the Edg-1, platelet-activating factor (PAF), and EP3β PGE2 receptors had similar binding affinity and biological activity as the wild types (*12*, *36*, *37*).

N-Glycosylation of hKOR and Receptor Regulation. Elimination of *N*-glycosylation of hKOR by N25/39Q mutation enhanced U50,488H-induced receptor phosphorylation, de-

sensitization, internalization, and downregulation (Figures 5 and 6). We have shown previously that internalization of the hKOR occurs via GRK-, β-arrestin-, and dynamin-dependent pathways and likely involves clathrin-coated vesicles (*28*) and that internalization of the hKOR is required for downregulation (*22*). Lacking *N*-glycosylation may affect downstream steps involved in agonist-induced receptor regulation, in addition to hKOR phosphorylation. In contrast to our observation, *N*-glycosylation is not involved in agonist-induced internalization of several receptors, including the bradykinin B2 and Edg-1 receptors (*12*, *38*). In addition, elimination of *N*-glycosylation of the δ opioid receptor by tunicamycin treatment did not affect agonist-induced receptor downregulation (*13*).

Differences between Tunicamycin Treatment and N25/39Q Mutations. Both 43- and 38-kDa bands were observed following treatment with tunicamycin and pulse labeling (Figure 2B), which are similar to the western blotting result of the double mutant. However, there was only a faint 38-kDa band observed following prolonged treatment with tunicamycin (Figure 2A), which may be due to conversion to the 43-kDa band and degradation of the 38-kDa band by ER-associated degradation. The reasons why prolonged tunicamycin treatment resulted in less 38-kDa band than N25/39Q mutations are not clear. It may be related to the differences that tunicamycin blocked *N*-linked glycosylation of *all* proteins, whereas N25/39Q mutations only eliminate *N*-linked glycosylation of the hKOR.

M_r of the hKOR Core Polypeptide. Treatment of the hKOR with Endo H or PNGase F or incubation of cells with tunicamycin resulted in generation of a 38-kDa form of the hKOR (Figures 1B,C and 2B). In addition, the N25/39Q mutant migrates as 38- and 43-kDa bands (Figure 3). These results taken together indicate that the 38-kDa band represents the core polypeptide of the hKOR. The M_r of 38 kDa for the core polypeptide is smaller than the predicted molecular mass of 42 kDa based on the amino acid sequence. Frank and Rodbard (*39*) previously demonstrated that even with optimal solubilization and disaggregation, the apparent molecular mass of rhodopsin, determined with SDS−PAGE using soluble protein standards, deviated substantially from the predicted molecular mass. This is most likely due to glycosylation and the highly hydrophobic nature of the hKOR (for a review, see ref *40*). The hydrophobic nature of the receptor may result in incomplete denaturation and disruption of disulfide bond(s). Glycosylation and high hydrophobicity of the receptor may lead to uncertainties regarding SDS binding.

In conclusion, the hKOR expressed in CHO cells is synthesized and cotranslationally *N*-glycosylated at N25 and N39 in the ER as the 45-kDa form, followed by trimming and further *N*-glycosylation and *O*-glycosylation in the Golgi to become 55 kDa, which is transported to plasma membranes. *N*-Glycosylation slows trafficking of the hKOR along the ER−Golgi−plasma membrane pathway but greatly enhances stability of the receptors proteins. *N*-Glycosylation also reduced agonist-induced regulation of the hKOR.

REFERENCES

1. Pasternak, G. W. (1988) *The Opiate Receptors*, Humana Press, Clifton, NJ.
2. Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J. (1988) Anatomy of CNS opioid receptors, *Trends Neurosci. 11*, 308−314.
3. von Voigtlander, P. F., Lahti, R. A., and Ludens, J. H. (1983) U-50,488: a selective and structurally novel non-Mu (kappa) opioid agonist, *J. Pharmacol. Exp. Ther. 224*, 7−12.
4. Dykstra, L. A., Gmerek, D. E., Winger, G., and Woods, J. H. (1987) Kappa opioids in Rhesus monkeys. I. diuresis, sedation, analgesia and discriminative stimulus effects, *J. Pharmacol. Exp. Ther. 242*, 413−420.
5. Pfeiffer, A., Brantl, V., Herz, A., and Emrich, H. M. (1986) Psychotomimesis mediated by kappa opiate receptors, *Science 233*, 774−776.
6. Law, P.-Y., Wong, Y. H., and Loh, H. H. (2000) Molecular mechanisms and regulation of opioid receptor signaling, *Annu. Rev. Pharmacol. Toxicol. 40*, 389−430.
7. Knapp, R. J., Malatynska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V. J., Roeske, W. R., and Yamamura, H. I. (1995) Molecular biology and pharmacology of cloned opioid receptors, *FASEB J. 9*, 516−525.
8. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) *Molecular Biology of the Cell*, Garland Science, New York.
9. Hawtin, S. R., Davies, A. R., Matthews, G., and Wheatley, M. (2001) Identification of the glycosylation sites utilized on the V1a vasopressin receptor and assessment of their role in receptor signalling and expression, *Biochem. J. 357*, 73−81.
10. Karpa, K. D., Lidow, M. S., Pickering, M. T., Levenson, R., and Bergson, C. (1999) N-linked glycosylation is required for plasma membrane localization of D5, but not D1, dopamine receptors in transfected mammalian cells, *Mol. Pharmacol. 56*, 1071−1078.
11. Mialet-Perez, J., Green, S. A., Miller, W. E., and Liggett, S. B. (2004) A primate-dominant third glycosylation site of the beta2-adrenergic receptor routes receptors to degradation during agonist regulation, *J. Biol. Chem. 279*, 38603−38607.
12. Kohno, T., Wada, A., and Igarashi, Y. (2002) N-glycans of sphingosine 1-phosphate receptor Edg-1 regulate ligand-induced receptor internalization, *FASEB J. 16*, 983−992.
13. Law, P. Y., Ungar, H. G., Hom, D. S., and Loh, H. H. (1985) Effects of cycloheximide and tunicamycin on opiate receptor activities in neuroblastoma X glioma NG108-15 hybrid cells, *Biochem. Pharmacol. 34*, 9−17.
14. Buhlmann, N., Aldecoa, A., Leuthauser, K., Gujer, R., Muff, R., Fischer, J. A., and Born, W. (2000) Glycosylation of the calcitonin receptor-like receptor at Asn(60) or Asn(112) is important for cell surface expression, *FEBS Lett. 486*, 320−324.
15. Compton, S. J., Sandhu, S., Wijesuriya, S. J., and Hollenberg, M. D. (2002) Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling, *Biochem. J. 368*, 495−505.
16. Zhong, X., Kriz, R., Seehra, J., and Kumar, R. (2004) N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression, *FEBS Lett. 562*, 111−117.
17. Mody, N., Hermans, E., Nahorski, S. R., and Challiss, R. A. (1999) Inhibition of N-linked glycosylation of the human type 1alpha metabotropic glutamate receptor by tunicamycin: effects on cell-surface receptor expression and function, *Neuropharmacology 38*, 1485−1492.
18. Xu, W., Chen, C., Huang; P., Li, J., de Riel, J. K., Javitch, J. A., and Liu-Chen, L.-Y. (2000) The conserved cysteine 7.38 residue is differentially accessible in the binding-site crevices of the mu, delta, and kappa opioid receptors, *Biochemistry 39*, 13904−13915.
19. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, *Nucleic Acids Res. 16*, 7351−7367.
20. Li, J., Li, J.-G., Chen, C., Zhang, F., and Liu-Chen, L.-Y. (2002) Molecular basis of differences in (−)(trans)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide-induced desensitization and phosphorylation between human and rat κ-opioid receptors expressed in Chinese hamster ovary cells, *Mol. Pharmacol. 61*, 73−84.
21. Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor, *J. Biol. Chem. 275*, 13727−13736.

22. Li, J.-G., Benovic, J. L., and Liu-Chen, L.-Y. (2000) Mechanisms of agonist-induced down-regulation of the human kappa-opioid receptor: internalization is required for down-regulation, *Mol. Pharmacol. 58*, 795−801.
23. Chen, Y., Chen, C., Wang, Y., and Liu-Chen, L. Y. (2006) Ligands regulate cell surface level of the human {kappa} opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: Differential effects of nonpeptide and peptide agonists, *J. Pharmacol. Exp. Ther. 319*, 765−775.
24. Petaja-Repo, U. E., Hogue, M., Bhalla, S., Laperriere, A., Morello, J. P., and Bouvier, M. (2002) Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation, *EMBO J. 21*, 1628−1637.
25. Chen, C., Li, J. G., Chen, Y., Huang, P., Wang, Y., and Liu-Chen, L. Y. (2006) GEC1 interacts with the kappa opioid receptor and enhances expression of the receptor, *J. Biol. Chem. 281*, 7983−7993.
26. Tallarida, R. J., and Murray, R. B. (1987) *Manual of Pharmacologic Calculations with Computer Programs*, Springer-Verlag, New York.
27. Li, J. G., Zhang, F., Jin, X. L., and Liu-Chen, L.-Y. (2003) Differential regulation of the human kappa opioid receptor by agonists: etorphine and levorphanol reduced dynorphin A- and U50,488H-induced internalization and phosphorylation, *J. Pharmacol. Exp. Ther. 305*, 531−540.
28. Li, J.-G., Luo, L. Y., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L.-Y. (1999) U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation, *J. Biol. Chem. 274*, 12087−12094.
29. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid, *Anal. Biochem. 150*, 76−85.
30. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. H., Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases, *Anal. Biochem. 180*, 195−204.
31. Umemoto, J., Bhavanandan, V. P., and Davidson, E. A. (1977) Purification and properties of an endo-alpha-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumoniae*, *J. Biol. Chem. 252*, 8609−8614.
32. Liu-Chen, L.-Y., Chen, C., and Phillips, C. A. (1993) Beta-[^{3}H]-funaltrexamine-labeled mu-opioid receptors: species variations in molecular mass and glycosylation by complex-type, N-linked oligosaccharides, *Mol. Pharmacol. 44*, 749−756.
33. Hebert, D. N., Garman, S. C., and Molinari, M. (2005) The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags, *Trends Cell Biol. 15*, 364−370.
34. Ohtsubo, K., Takamatsu, S., Minowa, M. T., Yoshida, A., Takeuchi, M., and Marth, J. D. (2005) Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes, *Cell 123*, 1307−1321.
35. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins, *EMBO J. 17*, 1919−1929.
36. Garcia, R. C., Cundell, D. R., Tuomanen, E. I., Kolakowski, L. F., Jr., Gerard, C., and Gerard, N. P. (1995) The role of N-glycosylation for functional expression of the human platelet-activating factor receptor. Glycosylation is required for efficient membrane trafficking, *J. Biol. Chem. 270*, 25178−25184.
37. Boer, U., Neuschafer-Rube, F., Moller, U., and Puschel, G. P. (2000) Requirement of N-glycosylation of the prostaglandin E2 receptor EP3beta for correct sorting to the plasma membrane but not for correct folding, *Biochem. J. 350* (Part 3), 839−847.
38. Michineau, S., Muller, L., Pizard, A., Alhenc-Gelas, F., and Rajerison, R. M. (2004) N-linked glycosylation of the human bradykinin B2 receptor is required for optimal cell-surface expression and coupling, *Biol. Chem. 385*, 49−57.
39. Frank, R. N., and Rodbard, D. (1975) Precision of sodium dodecyl sulfate-polyacrylamide-gel electrophoresis for the molecular weight estimation of a membrane glycoprotein: studies on bovine rhodopsin, *Arch. Biochem. Biophys. 171*, 1−13.
40. Hjelmeland, L. M., and Chrambach, A. (1981) Electrophoresis and electrofocusing in detergent containing Media−A discussion of basic concepts, *Electrophoresis 2*, 1−11.

BI700443J