GEC1 Interacts with the \( \kappa \) Opioid Receptor and Enhances Expression of the Receptor*

We identified a truncated form (38–117) of GEC1 that interacts with the C-tail of the human \( \kappa \) opioid receptor (hKOR) by yeast two-hybrid screening. GEC1-(38–117) did not interact with the C-tail of the \( \mu \) or \( \delta \) opioid receptors. GEC1, a 117-amino acid protein (Pellerin, I., Vuillermoz, C., Jouvenot, M., Ordener, C., Royez, M., and Adesil, G. L. (1993) *Mol. Cell Endocrinol.* 90, R17–R21), is highly homologous to GABARAP, GATE-16, and Apg8/aut7, all members of the microtubule associated protein (MAP) family. In pull-down assays, GST-GEC1 interacted directly with the hKOR C-tail, full-length hKOR, and tubulin. When expressed in Chinese hamster ovary (CHO) cells, GEC1 co-immunoprecipitated with FLAG-hKOR. Expression of GEC1 greatly increased total cell-surface KOR but not \( \mu \) or \( \delta \) opioid receptors. GEC1 expression slightly reduced U50,488H-promoted down-regulation, without affecting ligand binding affinity, receptor-G protein coupling, or U50,488H-induced desensitization and internalization. HA-GEC1 expressed in CHO cells was localized in the Golgi apparatus and endoplasmic reticulum (ER). When cells were pulsed with \([35S]\)Met/Cys, GEC1 expression enhanced the level of the mature form (55-kDa band) of FLAG-hKOR at 4, 8, and 22 h after pulse without affecting the precursors (39- and 45-kDa bands), indicating that GEC1 facilitates trafficking of FLAG-hKOR from the ER/Golgi to plasma membranes. GEC1 interacted with \( \gamma \)-ethylmaleimide-sensitive factor (NSF) in pull-down assays and co-immunoprecipitated with NSF in rat brain extracts. The interaction with NSF may contribute to GEC1 effects. This is the first report on biological functions of GEC1 and the first demonstration that a GPCR interacts with a protein of the MAP family. The interaction is important for trafficking of the receptor in the biosynthesis pathway.

Activation of \( \kappa \) opioid receptors in vivo produces many effects, including antinociception (1, 2), psychotomimosis (2, 3), and water diuresis (1, 2). At the cellular level, \( \kappa \) opioid receptors are coupled through pertussis toxin-sensitive G proteins to affect a variety of effectors, which include adenylate cyclase, potassium, and calcium channel and the p42/p44 mitogen-activated protein kinase (for a review, see Ref. 5). Follow-
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across the species cloned to date, including frog (AAH72921), mouse (NP_066615), guinea pig (AAL23264), and human (Q9H0R8), indicating that GEC1 is highly conserved in evolution. GEC1 mRNA is widely distributed in mouse and human tissues, with the expression levels being very high in the brain, heart, peripheral blood leukocytes, liver, kidney, placenta, and skeletal muscle (1, 13, 25). Nemos et al. (25) found that GEC1 mRNA was higher than GABARAP mRNA in the human central nervous system. GABARAP, GATE-16, and MAP1-LC3 have also been found to be widely distributed, including in the brain, liver, kidney, and heart (15, 16, 26, 27). The wide distribution of this family of proteins suggests that they have important biological functions in cells.

We hypothesized that GEC1 played an important role in the trafficking of the κ opioid receptor. In this study, we characterized the interaction between GEC1 and the hκOR and investigated the functional significance of this interaction on expression, signal transduction, and regulation of the receptor.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening

Yeast two-hybrid screening was conducted with the MATCHMAKER GAL4 Two-hybrid System 3 (BD Biosciences Clontech, Palo Alto, CA). The DNA fragment encoding amino acids 334–380 of the hκOR C-terminal domain (DENFKCRFDRFCPPLMRQERTSRTYQTDSPAY-LRQDRGMKVP) was inserted into pGKT7, the GAL4 binding domain plasmid (bait). The bait construct was transformed into AH109 yeast strain, and the transformants were selected on SD/-Trp plates. A human brain cDNA library constructed in the GAL4 activation domain vector pACT2 and pretransformed into yeast host strain Y187 (prey) was purchased from BD Biosciences Clontech (Palo Alto, CA). The AH109 bait transformant and the Y187 library host were mated in 2× YPDA medium (2% yeast extract, 4% peptone, 4% glucose, 0.006% adenine) for 24 h to give rise to diploids containing both bait and library constructs. The mating efficiency was measured at ~20%, which translated into ~2 million clones screened.

The complete mating culture was plated on 150-mm plates containing three drop-out medium (SD/-Trp/-Leu/-His/-Ade, QDO). The positive clones were re-streaked twice on four-drop-out media with X-a5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) added to test for α-galactosidase expression. The cDNAs from clones selected were isolated using the QiAamp DNA miniprep kit (Qiagen Co., Valencia, CA). The library vectors were rescued by transforming into Escherichia coli, and the isolated DNAs were used to co-transform with bait DNA into AH109 for further verification of protein-protein interaction. The library inserts in positive clones were amplified by PCR, and the fragments were sequenced, analyzed, and searched against GenBank entries (non-redundant, expressed sequence tags, and genome survey sequence data bases). The inserts from selected positive clones were subcloned into the mammalian expression vector pCMV containing the hemagglutinin (HA) epitope for transfection into CHO cells stably expressing FLAG-hκOR.

Similar to the hκOR C-tail construction, the C-tails of human µ, δ, and rat κ opioid receptors (hMOR, hDOR, and rκOR, Fig. 1B) and the truncated form hκOR Ct-345 were inserted into pGBK7 and transformed into the AH109 strain to generate bait clones. The GABARAP cDNA was a generous gift from Dr. Richard Olsen of UCLA. GABARAP (38–117) was cloned into pGAD7T and transformed into Y187. The bait transformants were mated with transformants of GEC1 (38–117) or GABARAP (38–117). The mating cultures were plated on SD/-Trp/-Leu, SD/TDO, and SD/QDO plates. The plates were incubated at 30°C for 7 days before the colony numbers were counted. The interaction strengths were evaluated and presented as the percent-age of the colony forming units (CFU) on TDO or QDO plates over those on -Trp/-Leu plates (percentage of total CFU, Fig. 1B).

Assessment of Direct Interactions between GEC1 and hKOR-C-tail or Tubulin by Pull-down Techniques

cDNA Construction and Generation of GST Fusion Proteins—The full-length cDNA of GEC1 was obtained from total RNA of HEK293 cells by reverse transcription-PCR. Three GST fusion proteins were used in the studies: GST-GEC1, the GST receptor C-tails of hKOR and hDOR. The cDNA constructs of the GST fusion proteins of receptor C-tails and GEC1 were generated in the pGEX-4T-1 bacterial expression system (Amersham Biosciences), transformed into E. coli BL21-CodonPlus (DE3)-RP (Stratagene, La Jolla, CA). GST receptor C-tails, GST-GEC1, or GST proteins were adsorbed onto glutathione-Sepharose 4B beads and washed three times with phosphate-buffered saline for pull-down analysis.

cDNA Construction and Purification of His6/S-Tag-GEC1—The full-length cDNA of GEC1 was cloned into the pET-30a (+) bacterial expression vector (Novagen Co., Madison, WI), which carries an N-terminal His tag and an S-tag. The construct was transformed into E. coli BL21(DE3), and the protein was adsorbed onto ProBond nickel-chelating resin (Invitrogen). The His6 tag fusion proteins were eluted from the beads with 0.5 M imidazole/500 mM NaCl/50 mM sodium phosphate buffer, pH 8.0, at room temperature for 20 min. Imidazole was removed via multiple rounds of concentration and dilution using Microcon centrifugal filter devices (Millipore, Bedford, MA) with TBS-T buffer (25 mM Tris/150 mM NaCl/0.1% Tween 20, pH 7.4).

Interaction of Receptor-C-tails with Purified GEC1—GST receptor C-tail fusion proteins or GST (control) bound to glutathione-Sepharose 4B beads (~20 μg of protein/20 μl of resin) were incubated with purified His6/S-tag-GEC1 (20 μg) in 1 ml of TBS-T buffer (25 mM Tris/150 mM NaCl/0.1% Tween 20, pH 7.4) containing 3% bovine serum albumin at 4°C with end-to-end rotation for 1 h. The beads were washed twice with ice-cold 3% bovine serum albumin in TBS-T buffer and three times with TBS-T buffer to remove nonspecific binding. The beads were then incubated with 40 μl of 2× Laemmli sample buffer (4% SDS, 0.1 M dithiothreitol, 20% glycerol, 62.5 μg Tris, pH 6.8) to dissociate proteins from beads and centrifuged to remove beads. The supernatants (20 μl each) were subjected to 15% SDS-PAGE, and the proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore), which was rinsed three times with TBS-T buffer and blocked with 2% nonfat dry milk in TBS-T buffer. The His6/S-tag-GEC1 was detected by blotting with S-protein horseradish peroxidase conjugate (Novagen) at 1:5000 dilution, followed by enhanced chemiluminescence reagents (Pierce). The membranes was also stained with 0.1% Ponceau S in 5% acetic acid to reveal the relative loading amounts of the GST (26 kDa) and GST receptor C-tails (34–35 kDa).

Interaction of GEC1 with Tubulin—GST or GST-GEC1 fusion protein was loaded onto glutathione-Sepharose 4B beads and incubated with soluble tubulin (PC-tubulin, Cytoskeleton, Denver, CO), in buffer A (20 mM Tris, pH 7.5, 5 mM MgCl2, 2 mM CaCl2, 1 mM dithiothreitol, 1% Triton X-100, and 1% nonfat milk) at room temperature for 1 h or overnight. Beads were washed extensively, and bound proteins were eluted with 2× Laemmli sample buffer. The proteins were resolved on SDS-PAGE and visualized by immunostaining with monoclonal anti-β-tubulin (Sigma-Aldrich) at 1:1000 followed by enhanced chemiluminescence reagents (21).

Stable Expression of Receptors in CHO Cells

CHO cells stably expressing FLAG-hKOR, HA-rMOR, FLAG-mDOR, hKOR, and rκOR were established previously (28). The tras
cated form, FLAG-hKOR-Ct-345 (with a 35-amino acid deletion from the C-terminal domain, Fig. 1B), was stably transfected into CHO cells similarly. These cells were designated as CHO-sFLAG-hKOR, CHO-HA-rMOR, CHO-sFLAG-mDOR, CHO-hKOR, CHO-rKOR, and CHO-sFLAG-hKOR Ct-345, respectively. The sFLAG-hKOR and sFLAG-mDOR constructs have a signal peptide preceding the FLAG epitope tag at N terminus, which is cleaved off N-terminal to the FLAG epitope by ER constructs have a signal peptide preceding the FLAG epitope tag at N terminus, which is cleaved off N-terminal to the FLAG epitope by ER processing. The signal peptide has been shown to enhance expression of the B2-adrenergic receptor (29). The CHO receptor cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 HAM supplemented with 10% fetal calf serum, 0.5 mg/ml Geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO2, and 95% air at 37 °C. These stable cell lines were used throughout this study in all experiments involved GEC1 transfection.

**Transient Expression of GEC1 or GEC1-(38–117) in CHO Cells**

Full-length GEC1 cDNA was inserted into the EcoRI/XhoI sites of the vector pcDNA3.1/Hygro (Invitrogen) with HA epitope added 5’ to the initiation codon of GEC1 for expression of HA-GEC1. Untagged wild-type GEC1 cDNA was also inserted into the EcoRI/XhoI sites of pcDNA3.1/Hygro. CHO-sFLAG-hKOR cells were transiently transfected with 10 μg/100-mm dish of HA-GEC1 or GEC1, HA-GEC1-(38–117) cDNA constructs or the vector pcDNA3 (control) by use of Lipofectamine (40 μl, Invitrogen) following the manufacturer’s instructions. About 40 h after transfection, cells were used for experiments.

**Pull-down Assay of GST-GEC1 with FLAG-hKOR or NSF in CHO Cells**

GST or GST-GEA1 fusion protein was loaded on glutathione-Sepharose 4B beads as described above. A 100-mm dish of CHO-sFLAG-hKOR cells at 90% confluent were harvested and solubilized in 1 ml of TTSEC buffer (2% Triton X-100/50 mM Tris HCl/150 mM NaCl/5 mM EDTA/mixture tablet of protease inhibitor (Roche Applied Science) (1 tablet/10 ml), pH 7.4) for 1 h at 4 °C and centrifuged at 100,000 × g for 20 min. An aliquot of 500-μl supernatant was incubated with GST-GEC1 or GST pre-loaded glutathione-Sepharose 4B beads on an end-to-end rocker for 1 h at 4 °C, washed, and SDS-PAGE was performed. Immunoblotting was performed with mouse anti-NSF antibody (Stressgen Biotechnologies, Inc., San Diego, CA) for NSF and rabbit anti-FLAG antibody (F7425, Sigma-Aldrich Co.) for FLAG-hKOR.

**Co-immunoprecipitation of FLAG-hKOR and GEC1**

This was performed according to our published procedure (30). Briefly, CHO-sFLAG-hKOR, CHO-sFLAG-mDOR, or CHO cells transiently transfected with GEC1 or HA-GEC1 were solubilized with TTSEC buffer for 1 h at 4 °C and centrifuged at 100,000 × g for 1 h. The supernatants were incubated for 1.5 h at 4 °C with agarose beads conjugated with M2 monoclonal anti-FLAG antibody (M2-Agarose, Sigma-Aldrich). The immunoprecipitated materials were washed three times with TTSEC and dissociated in 2× Laemml sample buffer with 100 mM dithiothreitol. Samples were subjected to SDS-PAGE and transferred onto Immobilon membranes. Membranes were treated with blocking solution, blotted with a rabbit antibody against GEC1 (PA629p) followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and enhanced chemiluminescence reagents (Pierce). FLAG-hKOR was blotted with rabbit anti-FLAG antibody (F7425, Sigma-Aldrich) after stripping of the same Immobilon membranes.

**Immunoblotting of Receptors**

Immunoblotting was performed to examine the expression of FLAG-hKOR, FLAG-mDOR, and HA-rMOR proteins as described previously (28). CHO-sFLAG-hKOR, CHO-sFLAG-mDOR, and CHO-HA-rMOR cells were solubilized with 2× Laemmli sample buffer, subjected to SDS-PAGE, and transferred onto Immobilon membranes. Membranes were treated with blocking solution, incubated with a rabbit antibody against FLAG (F7425) for FLAG receptors and a monoclonal antibody against HA (HA.11, Covance, Princeton, NJ) for HA-rMOR followed by goat anti-rabbit or -mouse IgG conjugated with horseradish peroxidase and then by enhanced chemiluminescence reagents. Images were captured by FUJIFILM LAS1000 plus system and quantitated using the ImageGauge software (version 4.1, Fuji Photo Film Co., Ltd.).

**Quantitation of Receptor Down-regulation by Western Blot**

HA-GEC1, HA-GEC1-(38–117), or the vector were transiently transfected into CHO-sFLAG-hKOR cells, transferred into 12-well plates 24 h after transfection, and cultured overnight. Cells were treated with or without 1 mM U50,488H at 37 °C for 4 h, and immunoblotting was performed as described above (31).

**Receptor Binding**

Receptor binding to FLAG-hKOR, FLAG-mDOR, HA-rMOR, and FLAG-hKOR Ct-345 expressed in CHO cells was conducted in intact cells as we described previously (32). Binding was conducted with 1 nM [3H]diprenorphine in Kreb’s buffer at 25 °C for 60 min in duplicate in a volume of 1 ml with 2 × 105 cells. Naloxone (10 μM) was used to define nonspecific binding.

**[35S]GTPγS Binding and U50,488H-induced Desensitization**

Membrane preparations and [35S]GTPγS binding were performed according to Li et al. (28). About 10 μg of membrane protein was incubated with 15 μM GDP and 0.2 μM [35S]GTPγS in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, and 0.1% bovine serum albumin, pH7.4) with or without different concentrations of U50,488H, in a final volume of 0.5 ml at 30 °C for 60 min. For desensitization experiments, cells were treated with or without 1 mM U50,488H for 60 min and, after extensive washing, membranes were prepared in the presence of phosphatase inhibitors (10 mM sodium fluoride and 10 mM tetrasodium pyrophosphate) (33).

**Quantitation of Surface Receptor and Receptor Internalization by Fluorescence Flow Cytometry**

CHO receptor cells transiently transfected with HA-GEC1, GEC1, HA-GEC1-(38–117), or the vector were transferred into 12-well plates ~20 h after transfection and cultured overnight. For internalization, CHO-sFLAG-hKOR cells were treated with vehicle or 1 μM U50,488H for 30 min at 37 °C, washed, and lifted off plates. Cell-surface FLAG-hKOR, FLAG-mDOR, and FLAG-hKOR Ct-345 were labeled with M1 anti-FLAG antibody (1 μg/ml, Sigma-Aldrich), and HA-rMOR was labeled with HA.11 anti-HA antibody followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (1 μg/ml, Molecular Probes). Immunoreactivity of cell-surface receptor was quantitated by fluorescence flow cytometry (FACSscan, BD Biosciences, San Jose, CA) according to our published procedure (34).

**Deglycosylation of hKOR by Endoglycosidase H**

This experiment was performed according to a procedure modified from that of Petäjä-Repo et al. (35). Briefly, the CHO-sFLAG-hKOR cells were washed with phosphate-buffered saline, harvested, sonicated, and centrifuged at 27,000 × g for 20 min. Pelleted crude membranes were washed twice with 50 mM Tris-HCl buffer/1 mM EGTA/10 mM
leupeptin (pH 7.4) and stored at −80°C. Thawed membranes were washed with lysis buffer (50 mM sodium phosphate, pH 5.5, 50 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-phenanthroline, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine) and pelleted by centrifugation at 27,000 × g for 20 min. Membranes were solubilized in lysis buffer containing 0.5% n-dodecyl-β-D-maltoside (w/v) at 1 mg of membrane protein/ml for 60 min at 4°C and centrifuged at 100,000 × g for 60 min at 4°C. The supernatant was incubated with Endo H (Roche Applied Science) at a final concentration of 20 μg/ml for 1 h at 37°C. The reaction was terminated by addition of 2× Laemmli sample buffer. The sample was subjected to SDS-PAGE, and immunoblotting of FLAG-hKOR was performed as described above.

**Pulse-chase Experiments on FLAG-hKOR Protein**

**Cell Culture and Transfection**—CHO-sFLAG-hKOR cells in growth phase were subcultured into 6-well plates at 3 × 10⁵ cells/well and allowed to grow to complete medium (Dulbecco’s modified Eagle’s medium/F-12, 10% fetal bovine serum, 0.5 mg of G418/ml) for 24 h. Cells were washed once with OPTI-MEM (Invitrogen) and preincubated in 0.8 ml of OPTI-MEM/well for at least 1 h before transfection. The transfection complex was prepared by mixing 10 μg of GEC1-pcDNA3.1 or control vector with 40 μl of Lipofectamine (Invitrogen) in 1.2 ml of OPTI-MEM. After 20-min incubation at room temperature, the mixture was dispersed into the 6-well plate at 0.2 ml/well (total of 1 ml/well) and incubated for 12–16 h. Medium was replaced with 2 ml/well fresh OPTI-MEM supplemented with 10% fetal bovine serum and incubated further for a total of 40 h after transfection. OPTI-MEM was antibiotics-free at all times. The transfection efficiency was titrated down to 0.025 μg of HA-GECl/well (0.8 cm²) to yield just enough specific fluorescence to be detectable to avoid artifacts due to overexpression. After 24 h, the cells were fixed with methanol for 10 min followed by acetone for 1 min and incubated with blocking solution (5% normal goat serum in phosphate-buffered saline) for 30 min. For double staining, the cells were incubated with HA.11 mouse anti-HA antibody (1:1000, Covance) plus rabbit anti-calreticulin antibody (1:250, Abcam Inc., Cambridge, MA) or rabbit anti-HA (1:50, Zymed Laboratories, Inc., South San Francisco, CA) plus mouse anti-58K Golgi protein antibody (1:50, Abcam Inc.) for 2 h at room temperature. After washing, the cells were incubated with goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 (1:500) plus goat anti-rabbit IgG (H+L) conjugated with Texas Red (1:500, Molecular Probes) for 1 h at room temperature. The indirect immunofluorescence was visualized with Olympus FluoView 300 confocal microscopy fitted with a 60× oil objective. The confocal images were edited with Adobe Photoshop Elements (Adobe Systems Inc., San Jose, CA) for brightness and contrast adjustment and for annotations.

**Immunocytofluorescence of GEC1 in CHO Cells**

CHO cells cultured in 8-well chamber slides were transiently transfected with HA-GECl as described above. The amount of DNA transfected was titrated down to 0.025 μg of HA-GECl/well (0.8 cm²) to yield just enough specific fluorescence to be detectable to avoid artifacts due to overexpression. After 24 h, the cells were fixed with methanol for 10 min followed by acetone for 1 min and incubated with blocking solution (5% normal goat serum in phosphate-buffered saline) for 30 min. For double staining, the cells were incubated with HA.11 mouse anti-HA antibody (1:1000, Covance) plus rabbit anti-calreticulin antibody (1:250, Abcam Inc., Cambridge, MA) or rabbit anti-HA (1:50, Zymed Laboratories, Inc., South San Francisco, CA) plus mouse anti-58K Golgi protein antibody (1:50, Abcam Inc.) for 2 h at room temperature. After washing, the cells were incubated with goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 (1:500) plus goat anti-rabbit IgG (H+L) conjugated with Texas Red (1:500, Molecular Probes) for 1 h at room temperature. The indirect immunofluorescence was visualized with Olympus FluoView 300 confocal microscopy fitted with a 60× oil objective. The confocal images were edited with Adobe Photoshop Elements (Adobe Systems Inc., San Jose, CA) for brightness and contrast adjustment and for annotations.

**Co-immunoprecipitation of NSF and GEC1 in Rat Brain Homogenate**

Rat brains were homogenized on ice with 10 ml brain of TEC buffer (50 mM Tris, 5 mM EDTA and 1 tablet of mixture proteases inhibitors (Roche Applied Science), pH 7.5) using a 50-ml homogenizer, centrifuged at 20,000 × g for 15 min at 4°C. The pellet per brain was solubilized in 5 ml of TTSEC buffer for 1 h on an end-to-end rocker at 4°C and centrifuged at 120,000 × g for 1 h at 4°C, and the supernatant was filtered through a 0.2-μm filter (CENTREX MF, S&S Biopath Inc., Riviera beach, FL). An aliquot of 2-ml supernatants was incubated with 10 μg of purified anti-GECl antibody (PA629p) or pre-immunized serum (pre.PA629) for 1 h at 4°C followed by 50 μl of PANSORBIN for 1 h at 4°C. The immuno-complex was pelleted, washed three times with TTSEC buffer, and dissociated with 50 μl of Laemmli sample buffer. SDS-PAGE and Western blots were performed as described above except the antibodies used were mouse anti-NFS and anti-GECl antibodies.
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, Student’s t-test was performed. p < 0.05 was the level of significance in all statistical analyses.

RESULTS

**GEC1-(38–117) Specifically Associated with the C-tail of the KORs in a Yeast Two-hybrid Assay**—We performed yeast two-hybrid screening of a human brain cDNA library with the C-tail (334–380 fragment) of the hKOR as the bait. One of the 18 clones identified was the N-terminal truncated form of GEC1 (GEC1-(38–117)). The full-length cDNA of the human GEC1 was obtained by reverse transcription-PCR of RNA derived from HEK293 cells. The full-length GEC1 did not interact with the hKOR C-tail in the yeast two-hybrid assay (data not shown), presumably due to its binding to tubulin (see Fig. 3C), and hence microtubules, which may prevent AD-GEC1 fusion protein from entering the nucleus. Therefore, we used GEC1-(38–117) instead of the full-length GEC1 in yeast two-hybrid experiments.

To determine the specificity of association of GEC1-(38–117) with the hKOR C-tail among opioid receptors, we examined the interactions of GEC1-(38–117) with C-tails of human δ and μ opioid receptors and the rat KOR. GEC1-(38–117) interacted with the C-tails of the human δ opioid receptors and the truncated form of the hKOR, Ct-345 in yeast two-hybrid assay. Each of the C-tail constructs of the human δ and μ opioid receptors, and the rat KOR opioid receptor was transformed into the yeast AH109 strain. The GEC1-(38–117) or GABARAP-(38–117) construct was transformed into the mating partner strain Y187. The two strains were mated and plated on -Trp/-Leu plates. Aliquots that yielded similar numbers of colonies on -Trp/-Leu plates were cultured on -Trp/-Leu/-His (TDO) and -Trp/-Leu/-His/-Ade (QDO) plates. Interaction strength was calculated by the ratio of CFU on TDO or QDO over CFU on -Trp/-Leu plates, which is expressed as a percentage of total CFU. The experiments were performed three times with similar results. ND, not determined. Comparison of amino acid sequences of the C-tails is shown, and the numbers indicate those of the hKOR. - indicates amino acid identical to that of the hKOR.

| C-tails | GEC1(38–117) | GABARAP (38–117) |
|---------|-------------|-----------------|
| Human κ opioid receptor | 88±7 / 83±10 | 76±9 / 66±5 |
| Human μ opioid receptor | 0 / 0 | 0 / 0 |
| Human δ opioid receptor | 0 / 0 | 0 / 0 |
| Rat κ opioid receptor | 71±12 / 63±15 | ND |
| hKOR Ct-345 | 0 / 0 | ND |
| Control (pGBKNeg) | 0 / 0 | 0 / 0 |

**FIGURE 1.** A, the deduced amino acid sequence of GEC1 and its comparison with those of GABARAP, GATE-16, and the yeast protein Apg8/Aut7 (15–17). The GEC1 fragment-(38–117) identified in yeast two-hybrid screening to interact with the hKOR C-tail is underlined. -, amino acid identical to that of GEC1. B, GEC1-(38–117) and GABARAP-(38–117) interacted with the C-tails of human and rat KORs but not the C-tails of the human μ and δ opioid receptors and the truncated form of the hKOR, Ct-345 in yeast two-hybrid assay. Each of the C-tail constructs of the human δ and μ opioid receptors, and the rat κ opioid receptor was transformed into the yeast AH109 strain. The GEC1-(38–117) or GABARAP-(38–117) construct was transformed into the mating partner strain Y187. The two strains were mated and plated on -Trp/-Leu plates. Aliquots that yielded similar numbers of colonies on -Trp/-Leu plates were cultured on -Trp/-Leu/-His (TDO) and -Trp/-Leu/-His/-Ade (QDO) plates. Interaction strength was calculated by the ratio of CFU on TDO or QDO over CFU on -Trp/-Leu plates, which is expressed as a percentage of total CFU. The experiments were performed three times with similar results. ND, not determined. Comparison of amino acid sequences of the C-tails is shown, and the numbers indicate those of the hKOR. - indicates amino acid identical to that of the hKOR.
and rat KORs but not with those of the human δ and μ opioid receptors (Fig. 1B). When the C-terminal 35 amino acids were deleted from the hKOR C-tail (hKOR Ct-345), the interaction was abolished (Fig. 1B). Detailed interaction sites are being determined.

We also examined whether the C-tails of the opioid receptors interacted with the N-terminal truncated form of GABARAP, GABARAP-(38–117), in the yeast two-hybrid assay. The hKOR C-tail bound to GABARAP-(38–117) at 76% on TDO, 66% on QDO plates of total CFU (38–117), in the yeast two-hybrid assay. The hKOR C-tail bound to acted with the N-terminal truncated form of GABARAP, GABARAP-(38–117). Detailed interaction sites are being determined.

**FIGURE 2.** FLAG-hKOR co-immunoprecipitated with GEC1. GEC1 was transiently transfected into CHO-sFLAG-hKOR, CHO-sFLAG-mDOR, and CHO cells. Approximately 40 h later, cells were solubilized and immunoprecipitated with anti-FLAG M2-Agarose beads. Immunoblotting was performed using rabbit anti-GEC1 antibody (PA629p) for GEC1 and, after stripping, with rabbit anti-FLAG antibody for FLAG-hKOR and FLAG-mDOR as described under “Experimental Procedures.” Each figure represents one of three independent experiments performed with similar results.

**GEC1 Co-immunoprecipitated with the hKOR in Mammalian Cells**—We then investigated whether the hKOR could co-immunoprecipitate GEC1 in mammalian cells. GEC1/pcDNA3.1(Hygro) was expressed in CHO-sFLAG-hKOR, CHO-sFLAG-mDOR, and untransfected CHO cells. FLAG-hKOR and FLAG-mDOR were immunoprecipitated by use of M2 anti-FLAG antibody–Agarose beads (Fig. 2, upper panel). Immunoblotting with rabbit anti-GEC1 antibody (PA629p) revealed that GEC1 co-immunoprecipitated more preferably with FLAG-hKOR than with FLAG-mDOR, while no GEC1 co-precipitated in the absence of either receptor, as shown in the CHO cell control (Fig. 2, middle panel). These results indicate that the full-length hKOR interacts with GEC1 in CHO cells. Co-immunoprecipitation of HA-GEC1-(38–117) and FLAG-hKOR was also observed (data not shown).

**GEC1 Interacted Directly with the hKOR C-tail**—Next, we examined whether GEC1 bound directly to the hKOR C-tail using the pull-down techniques. GST, GST-hKOR C-tail, and GST-hDOR C-tail (Fig. 1B for specified amino acid sequences) and His6/S tag-GEC1 were expressed in E. coli and purified. As shown in Fig. 3A, the GST-hKOR C-tail bound GEC1 to a much greater extent than GST- hDOR C-tail, which showed similar binding as GST alone. These results indicate that GEC1 interacts directly with the hKOR C-tail but not with hDOR C-tail. We then determined if FLAG-hKOR expressed in CHO cells interacted with the GST-GEC1. CHO-sFLAG-hKOR cells were solubilized and pull-down experiments were performed. As shown in Fig. 3B, FLAG-hKOR, detected by FLAG antibody, bound to GST-GEC1, but not GST, indicating that the GEC1 binds FLAG-hKOR in CHO cell extracts.

**GEC1 bound Tubulin**—We examined whether GEC1 was associated with purified tubulin. CaCl2 was included in the binding buffer to keep tubulin in a soluble form. Tubulin interacted with GST-GEC1, but not with GST (Fig. 3C). Because tubulin used in the study is “PC-tubulin,” which is devoid of MAPs (21), these results demonstrate that GEC1 binds to soluble tubulin directly, but not mediated by other MAPs. We were unable to purify GEC1-(38–117), because it was retained in inclusion bodies in E. coli and thus had very low yield.

**GEC1 Enhanced Cell-Surface Receptors and Total Receptors of the KORs**—Expression of GEC1 or HA-GEC1 in CHO-sFLAG-hKOR cells increased the total FLAG-hKOR expression by ~90% (Fig. 4A) and cell-surface FLAG-hKOR by ~130% (Fig. 4B) compared with the vector control, as determined by receptor binding. In contrast, GEC1 expression did not affect total and cell-surface levels of μ and δ opioid receptors. The expression level of the C-terminal deletion mutant, hKOR Ct-345, was not affected by GEC1 (Fig. 4, A and B). Expression of the truncated form, GEC1-(38–117), increased total hKOR numbers by...
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37.1 ± 3.4% (n = 3, mean ± S.E.), indicating that GEC1-(1–37) plays an important role in this effect. The presence of the HA epitope did not affect GEC1 effects (data not shown). Transfection of GEC1 and GEC1-(38–117) did not change the Kᵣ value of [³H]diprenorphine binding or the Kᵣ value of U50,488H binding to the hKOR (data not shown). Consistent with receptor binding results, transfection of GEC1 significantly increased the intensities of 55- and 45-kDa forms of FLAG-hKOR (Fig. 4C) but did not affect expression of the μ, δ, and the hKOR Ct-345 proteins (Fig. 4C). The hKOR construct used in this study has a cleavable signal peptide preceding the FLAG tag at N terminus, which enhances the ER insertion of newly synthesized β₂-adrenergic receptor (29). We examined whether the untagged wild-type hKOR and rKOR expression levels in CHO cells were affected by GEC1 transfection. GEC1 expression increased the total receptor binding of hKOR and rKOR by ~65% and ~57%, respectively (Fig. 4D), indicating that the signal peptide is not required for the enhancement to occur.

GEC1 Did Not Affect U50,488H-induced [³⁵S]GTPS Binding—Compared with the control, expression of HA-GEC1 and HA-GEC1-(38–117) in CHO-sFLAG-hKOR cells did not alter the EC₅₀ or Emax value of U50,488H in stimulating [³⁵S]GTPS binding (data not shown), indicating that GEC1 does not affect receptor-G protein coupling.

GEC1 Did Not Affect U50,488H-induced Desensitization or Internalization of the hKOR—Following incubation of cells with 1 μM U50,488H for 1 h, the Emax of U50,488H in promoting [³⁵S]GTPS binding was reduced with no change in the EC₅₀, indicating that the hKOR is desensitized, similar to what we observed previously (28, 33). Expression of HA-GEC1 and HA-GEC1-(38–117) in CHO-sf-hKOR cells did not affect U50,488H-induced desensitization (data not shown). In addition, pretreatment with 1 μM U50,488H for 30 min caused internalization of ~30% of FLAG-hKOR, similar to our previous reports (34). U50,488H promoted similar degree of FLAG-hKOR internalization in CHO-sFLAG-hKOR cells transfected with the vector and HA-GEC1.

GEC1 Reduced U50,488H-induced Down-regulation of the hKOR—Incubation of CHO-sFLAG-hKOR cells with U50,488H for 4 h resulted in ~25% reduction in 55-kDa band, similar to what we observed previously by receptor binding (33) and by immunoblotting (31). GEC1 expression reduced the degree of U50,488H-induced down-regulation of FLAG-hKOR to ~18% (Fig. 5).

GEC1 Was Localized in the Endoplasmic Reticulum and the Golgi Apparatus—Immunohistochemical staining of CHO-sFLAG-hKOR cells transfected with HA-GEC1 showed that HA-GEC1 located close to the nucleus with a punctate pattern (Fig. 6, middle panel, either green or red). GEC1 was co-localized with the 58-kDa Golgi protein, a marker for the Golgi apparatus, as shown by the yellowish color in the merged image (Fig. 6, upper right). In addition, when cells were double labeled with monoclonal anti-HA antibody and antibody against calreticulin, an ER marker, a yellowish color resulted after the images were merged (Fig. 6, lower right), indicating that ER contains GEC1.

GEC1 Enhanced Expression of Fully Glycosylated Forms of the hKOR—SDS-PAGE followed by immunoblotting with anti-FLAG antibody revealed that FLAG-hKOR expressed in CHO cells migrated as two bands of M₁₅ and M₄₅ kDa molecular mass (Fig. 7A). Treatment with Endo H cleaved the 45-kDa band to 39 kDa (Fig. 7A). Endo H selectively cleaves unprocessed high mannose type oligosaccharides from glycoproteins, but does not remove complex type fully processed glycans. Thus, these results indicate that the 45-kDa band

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*Figure 4. Expression of GEC1 increased (A) total receptor binding and (B) cell-surface receptor binding of FLAG-hKOR, but not the μ, δ, opioid receptor or the truncated form FLAG-hKOR Ct-345. GEC1 or the vector (control) was transiently transfected into CHO-sFLAG-hKOR cells, CHO-HA-rMOR, CHO-sFLAG-mDOR, or CHO-sFLAG-hKOR Ct-345 cells. Approximately 40 h later, receptor binding was performed with [³H]diprenorphine (~1 nM) to intact cells. For total receptors, 10 μM naloxone was used to define nonspecific binding. For cell-surface receptors 10 μM D-Pen-Cys-Tyr-o-Trp-D-Arg-D-Phe-Lys-NH₂, β-Penicillamine, α-Penicillamine, and dynorphin-(1–17) were used to define nonspecific binding for μ, δ, and κ receptors, respectively. Data are expressed as the percentage of the control. Each value represents the mean ± S.E. of three independent experiments. C, expression of GEC1 increased FLAG-hKOR, without affecting HA-rMOR, FLAG-mDOR, or FLAG-hKOR Ct-345. Transfection of GEC1 was performed as described in A. Approximately 40 h later, immunoblotting was performed for detection of μ, δ, and κ opioid receptors with antibodies against HA or FLAG. The figure represents one of the three experiments performed with similar results. D, expression of GEC1 increased total receptor binding of wild-type hKOR and wild-type rKOR. CHO cells stably transfected with untagged hKOR or rKOR were examined. Transfection of GEC1 and receptor binding assay were performed as described in A. Each value represents the mean ± S.E. of three independent experiments.*
represents ER and/or cis-Golgi glycosylated intermediates, and the 39-kDa band is the core polypeptide. In contrast, the 55-kDa band was not altered by Endo H treatment (Fig. 7A), demonstrating that the 55-kDa band represents the fully glycosylated form of the hKOR, which is located mostly in plasma membranes with some in the Golgi.

Pulse-chase experiments were performed to determine whether the elevated level of cell-surface receptors was due to enhanced insertion of the hKOR into or reduced removal of the hKOR from plasma membranes. Transfection of GEC1 significantly increased intensities of the 55-kDa form of the receptor at 4 h, 8 h, and 22 h after pulse, compared with transfection with the control vector (Fig. 7B). The peak level of the 55-kDa band was reached at 2 h for the control, but at 4 h for GEC1-transfected cells (Fig. 7C). The rate of decline in the intensity of the 55-kDa band was similar between GEC1-transfected and control cells, indicating that the half-life of fully glycosylated receptors is not changed by GEC1. There were no significant differences in the intensities of the 45- and 39-kDa forms between vector- and GEC1-transfected cells at any time point (Fig. 7C). These results indicate that GEC1 enhances maturation and
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E.G.C1 Interacts with the hKOR—Although GEC1 expression profoundly enhanced hKOR trafficking to plasma membrane, it co-immunoprecipitated with hKOR to a less extent (i.e. <1% of total GEC1 loaded, see Fig. 2) than what we observed for other proteins co-immunoprecipitated with hKOR, such as caveolin-11 and NHERF-1/EBP50 (30). This may be explained by the observation that GEC1 was found mainly in the Golgi and ER (Fig. 6 and Wang et al.),4 where only KOR in the biosynthesis pathway, a small fraction of total KOR, is co-localized with GEC1. Moreover, because GEC1 may function in membrane fusion (see below), it is likely to interact with KOR in a transient and dynamic fashion.

GEC1 interacted specifically with KORs among the opioid receptors. Alignment of C-tails of KORs, hMOR and hDOR (Fig. 1B), points to the C-terminal 37 amino acids of KORs (Phe-344 through Val-380) as the most likely region responsible for interaction with GEC1. We thus generated 35-amino acid truncated forms (Phe-346 to Val-380) of both the hKOR C-tail (hKOR Ct-345, Fig. 1B) and the FLAG-hKOR (FLAG-hKOR Ct-345, Fig. 4, A–C). Truncation of Phe-346 to Val-380 completely abolished the interaction of GEC1 with the receptor and the GEC1 enhancement of receptor expression level. The detailed amino acid sequences in both KOR and GEC1 responsible for this interaction are currently under investigation.

It should be noted that, because CHO cells were stably transfected with FLAG-hKOR in the vector pcDNA3, the transcription of the receptor was regulated by the constitutively active cytomegalovirus promoter. Therefore, it is not likely that GEC1 affects transcription of the receptor.

GEC1 Interacts with Tubulin, and This Interaction Is Important for Increasing Cell-Surface and Total hKORs—Our finding that GEC1 interacted directly with tubulin in pull-down assays (Fig. 4) is consistent with the report of Mansuy et al. (37) that GEC1 bound tubulin and enhanced tubulin assembly and microtubules bundling. Similarly, GABARAP has been found to interact with tubulin (15, 21, 38). The tubulin binding domain of GABARAP and GEC1 were identified to be within the N-terminal 22 amino acids (21, 37).

The x-ray crystal structure of GABARAP shows that it consists of an N-terminal basic helical region, which is involved in binding of tubulin and microtubules (21). The three-dimensional structure of GATE-16 and GABARAP are almost perfectly superimposable (39, 40). It is likely that the structure of GEC1 is similar to those of GATE-16 and GABARAP.

The full-length GEC1 increased both cell-surface and total receptors of the hKOR to a much greater extent than GEC1-(38–117), indicating that the N-terminal region (1–37) of GEC1 binding to tubulin, hence microtubules, plays an important role in these increases. This observation is similar to that of Wang et al. (21) that the tubulin binding region of GABARAP is important for its stimulatory effect on clustering of GABA receptors.

In yeast two-hybrid experiments, full-length GEC1 did not interact with the hKOR C-tail, although GEC1-(38–117) did, most likely due to its binding to microtubules, thus preventing it from entering nuclei. This is consistent with results of Wang et al. (15) that, in the yeast two-hybrid assay, GABARAP-(36–117) bound to the y2 subunit of the GABA receptor, but the full-length GABARAP did not.

GEC1 Is Localized in the ER and the Golgi Apparatus—HA-GEC1 expressed in CHO cells was localized in the ER and the Golgi apparatus (Fig. 3).}

3 W. Xu, S. I. Yoon, P. Huang, Y. Wang, P. L. G. Chong, and L. Y. Liu-Chen, manuscript submitted for publication.
4 Y. Wang, S. L. Dun, P. Huang, C. Chen, N. J. Dun, E. J. Van Bockstaele, and L. Y. Liu-Chen, manuscript submitted for publication.
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6) We have recently demonstrated using immunoelectron microscopy that GEC1 in neurons of the hypothalamus of the rat brain is localized in the ER > plasma membranes > cytosol > Golgi. Mansuy et al. (37) reported that GEC1-GFP or GFP-GEC1 transfected into HEK cells was localized in perinuclear vesicles; however, the organelles were not identified. In addition, GABARAP is enriched within the Golgi and postnysyncytial cisternae (20) and GATE-16 is associated with the Golgi (16). Because of the association of GEC1 with NSF and the known functions of GATE-16 and GABARAP, it is reasonable to postulate that GEC1 plays an important role in membrane fusion events, particularly along the ER to Golgi to plasma membrane pathway. The FLAG-hKOR cDNA used in the study has a cleavable modified influenza hemagglutinin signal sequence N-terminal to the FLAG epitope. The signal peptide has been shown to enhance expression of the \(\beta_2\)-adrenergic receptor and the action is due to more efficient translocation of the newly synthesized receptor into ER membranes (29).

GEC1 enhanced expression of FLAG-hKOR, indicating that GEC1 acts on a site or sites downstream of insertion of receptor into ER membranes. The enhancement in cell-surface FLAG-hKOR is attributed to increased maturation and insertion of the hKOR into plasma membranes but not reduced removal from plasma membranes. Thus, GEC1 most likely acts in the biosynthesis pathway to enhance the trafficking of the hKOR, leading to its increased cell surface expression.

GEC1 Interacts with NSF and the Role of NSF in Receptor Trafficking—We found that GEC1 interacted with NSF, a protein critical for intracellular membrane-trafficking events. GABARAP and GATE-16 have been shown to be associated with NSF (16, 20).

Recent reports have shown that interaction of receptors with NSF affects receptor trafficking. GluR2 has been shown to be associated with NSF, and this interaction is essential for cell-surface expression of GluR2-containing \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptors. Disrupting the interaction results in the functional elimination of \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptors at synapses (41, 42). Cong et al. (43) demonstrated that NSF bound to the \(\beta_2\)-adrenergic receptor and directed internalized \(\beta_2\)-adrenergic receptors to the recycling pathway. The association of GEC1 with NSF may contribute to the enhanced expression of the KOR. This hypothesis is currently being investigated. The amino acid sequence in GEC1 required for interacting with NSF is being determined.

GEC1 Reduces USO488H-induced Down-regulation—Expression of GEC1 reduced USO488H-induced hKOR down-regulation, without affecting internalization and desensitization. We have shown previously that USO488H-induced hKOR internalization is mediated by G protein-coupled receptor kinase-, \(\beta\)-arrestin-, and dynamin-dependent processes, likely involved clathrin-coated vesicles (32). Down-regulation involves, in addition, Rab5- and Rab7-mediated fusion of early endosomes, late endosomes, and lysosomes (44). Because the probable sites of action of GEC1 action are along the biosynthesis pathway, its effect on down-regulation are most likely secondary to enhanced cell-surface receptor levels, making the process limiting. Another possibility, although remote, that cannot be excluded is that GEC1 enhances receptor recycling, resulting in reduced down-regulation.

Expression of GEC1 did not affect USO488H-stimulated \(^{35}\)S)GTP\(\gamma\)S binding, indicating that GEC1 has no effect on hKOR-G protein coupling. GEC1 augmented cell-surface receptor expression without affecting agonist-induced \(^{35}\)S)GTP\(\gamma\)S binding, demonstrating the presence of a hKOR receptor reserve in GEC1-transfected cells so that activation of a portion of the receptors was sufficient to elicit maximal responses. Our result demonstrates that the receptor level does not always correlate with agonist-stimulated maximum response in an expression system. Similar observations have been reported by several groups. Law et al. (45) found that a 4-fold reduction of MOR level from 14 pmol/mg of protein to 2.8 pmol/mg of protein in HEK293 cells only decreased the etorphine-induced maximal inhibition of adenylyl cyclase activity by 17%. Whaley et al. (46) systematically explored the relationship of \(\beta_2\)-adrenergic receptor expression level to the \(V_{\text{max}}\), for adenylyl cyclase activation by epinephrine. They found that \(V_{\text{max}}\) was sharply increased when the receptor level increased from 0.005 to \(~0.050\) pmol/mg of protein and the \(V_{\text{max}}\) then reached a plateau at \(>0.050\) pmol of receptor/mg of protein. The stable cell line CHO-sFLAG-hKOR used in this study has a \(B_{\text{max}}\) of \(~1\) pmol/mg of protein.

GEC1 Levels—We have observed a moderate level of endogenous GEC1 in CHO cells. An attempt to reduce GEC1 levels by stable transfection of cells with small interference RNAs against GEC1 failed, due to massive cell death \(~1\) week after transfection. In addition, we transfected cells with GEC1 cDNA to establish stable cell lines expressing higher levels of GEC1. None of the cell lines isolated expressed higher levels of GEC1. It appears that cells with higher or lower GEC1 levels may survive for a short time, but not for long, suggesting that GEC1 may be tightly regulated.

GEC1 Analogs Are Involved in Intracellular Membrane Fusion—GABARAP (15), GATE-16, (16), and Apg8p/Aut7 and MAP1-LC3 (18) have all been implicated in membrane fusion processes. Like GEC1, all four proteins are shown to bind to tubulin, thus microtubules. GABARAP, GATE-16, and GEC1 are located in the Golgi and are associated with NSF. GABARAP and GATE-16 have very similar three-dimensional structures. GABARAP interacts with the \(\gamma_2\) subunit of the GABA\(_\text{A}\) receptor (15), and the interaction enhances clustering of the GABA\(_\text{A}\) receptor at the postsynaptic membrane and changes GABA\(_\text{A}\) receptor channel kinetics, ion conductance, and receptor regulation (23, 47). Expression of YFP-GABARAP increases the level of GABA\(_\text{A}\) receptor at the plasma membrane (48). GATE-16, highly expressed in brain (16), regulates intra-Golgi transport, through interactions with NSF, SNAP, and GIS-28 (16, 49, 50). The yeast protein Apgp8p/Aut7p is essential for delivery of autophagic vesicles to the vacuoles (51).

GEC1 Is Widely Distributed in the Central Nervous System—Our immunoblotting results showed that GEC1 was detected in all the regions of the rat central nervous system examined, with high levels in caudate putamen, nucleus accumbens, hypothalamus, hippocampus and thalamus, moderate levels in spinal cord, pons, olfactory bulb, and a low level in cerebellum. The widespread distribution of GEC1 was confirmed by immunohistochemistry of tissue sections. Nemos et al. (25) reported that a high level of GEC1 mRNA was present in the human brain and had broad distribution in brain regions. In contrast to GEC1, the \(\kappa\) opioid receptor has differential distribution in the rat brain, with high levels in claustrum, amygdala, olfactory tubercle, nucleus accumbens, caudate putamen, preoptic area, and hypothalamus (52). The broader localization of GEC1 implies that GEC1 may serve a similar role for many other molecules to facilitate their trafficking in the biosynthesis pathway. GEC1 was recently demonstrated to be associated with the \(\gamma_2\) subunit of GABA\(_\text{A}\) receptor (37). Along the same line, Green et al. (53) demonstrated that GABARAP interacted with the cytoplasmic domain of the transferrin receptor, indicating a role in non-neuronal cells and unrelated to the GABA\(_\text{A}\) receptor.

Regulation of GPCR Trafficking in the Biosynthesis Pathway—This has not been as extensively studied as trafficking in agonist-induced internalization and down-regulation pathways. For example, Berman et al. (54) reported that D1 dopamine receptor interacted with an ER membrane-associated protein, DRIF78, and expression of DRIF78 caused retention of the D1 receptor in the ER. Rhodopsin has been shown to interact with TcTex-1, a dynemin light-chain subunit, and this
interaction is necessary for the transport of post-Golgi rhodopsin-containing vesicles along the microtubules up to the outer segment (55). mGluR5 is associated with Homer proteins and sorted to dendrites by Homer1b, which remains in the intracellular compartment. Upon activation, Homer1a displaces Homer1b from mGluR5, which targets the receptor to the plasma membrane (56). Petaja-Repo et al. (35, 57) demonstrated that the majority of newly synthesized δ opioid receptor was retained in the ER, and cell-permeable δ ligands act as pharmacological chaperones to enhance maturation and cell-surface expression of the receptor. We now added GEC1 to the growing list of molecules regulating trafficking of GPCRs in the biosynthesis pathway.

Regulation of GEC1 mRNA by Estrogen—In guinea pig endometrial glandular epithelial cells GEC1 mRNA was increased by 2-fold following 2 h of estradiol-17β treatment (1). Estradiol treatment of the guinea pig for 24 h enhanced GEC1 mRNA by 2.7-fold in the preoptic area in the brain (58). The GEC1 gene contains a putative estrogen response element (GCGTCAAGTGTAGC) at 275 bp upstream from the ATG initiation codon (24).

In the superficial dorsal horn of the rat spinal cord, KOR immunoreactivity was significantly denser in estrus and proestrus females than in males (59). In addition, in female rats, δ opioid receptors have been shown to vary across the estrus cycle, being lower in diestrus than in proestrus or estrus (60). Because GEC1 is present in the superficial dorsal horn, whether GEC1 contributes to these differences needs further investigation.

Conclusion—Our findings strongly implicate GEC1 in the trafficking of the hKOR from ER to Golgi to plasma membranes. The widespread distribution of GEC1 mRNA (1, 13, 25) and GEC1 protein suggests that it may play a similar role for many proteins. Because estrogen up-regulates GEC1 mRNA (1, 58), GEC1 may be important in sex differences of many biological functions.

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