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Effects of C-terminal Modifications of GEC1 Protein and γ-Aminobutyric Acid Type A ($GABA_A$) Receptor-associated Protein (GABARAP), Two Microtubule-associated Proteins, on κ Opioid Receptor Expression*

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We demonstrated previously that GEC1, a member of the microtubule-associated protein (MAP) family, bound to the human κ opioid receptor (hKOPR) and promoted hKOPR cell surface expression by facilitating its trafficking along the secretory pathway. $GABA_A$ receptor-associated protein (GABARAP), a GEC1 analog, also enhanced KOPR expression, but to a lesser extent. The MAP family proteins undergo cleavage of their C-terminal residue(s), and the exposed conserved glycine forms conjugates with phosphatidylethanolamine, which associate with membranes. Here, we examined whether such modifications were required for GEC1 and GABARAP to enhance hKOPR expression. When transiently transfected into CHO or Neuro2A cells, GEC1 and GABARAP were cleaved at the C termini. G116A mutation alone or combined with deletion of Lys^{117} in GEC1 (GEC1-A) or Leu^{117} in GABARAP (GABARAP-A) blocked their C-terminal cleavage, indicating that the conserved Gly^{116} is necessary for C-terminal modification. The two GEC1 mutants enhanced hKOPR expression to similar extents as the wild-type GEC1; however, the two GABARAP mutants did not. Immunofluorescence studies showed that HA-GEC1, HA-GEC1-A, and HA-GABARAP were distributed in a punctate manner and co-localized with KOPR-EGFP in the Golgi apparatus, whereas HA-GABARAP-A did not. Pulldown assay of GST-KOPR-C-tail with HA-GEC1 or HA-GABARAP revealed that GEC1 had stronger association with KOPR-C-tail than GABARAP. These results suggest that because of its stronger binding for hKOPR, GEC1 is able to be recruited by hKOPR sufficiently without membrane association via its C-terminal modification; however, due to its weaker affinity for the hKOPR, GABARAP appears to require C-terminal modifications to enhance KOPR expression.

κ opioid receptor (KOPR)[2] is one of the three major types of opioid receptors mediating the effects of opioid drugs and endogenous peptides. The effects of KOPR activation *in vivo* include anti-nociception (especially for visceral chemical pain), anti-pruritic, water diuresis, and psychotomimetic effects (1). The KOPR agonist nalfurafine (TRK-820) is used clinically in Japan for the treatment of uremic pruritus in kidney dialysis patients (2). KOPR antagonists may be useful for curbing cocaine craving and as anti-anxiety drugs (3, 4). In addition, it has been proposed that KOPR agonists may be useful in treating mania, as antagonists as anti-depressants, and as partial agonists for the management of bipolar disorder (5).

We have demonstrated that the protein glandular epithelial cell 1 (GEC1) interacts directly with the C-terminal domain of the KOPR by hydrophobic interactions (6, 7). The interaction increases cell surface expression of the KOPR by enhancing the conversion of the glycosylated intermediates to fully glycosylated forms of the receptor, indicating facilitation of trafficking from the endoplasmic reticulum to Golgi to plasma membranes (7).

GEC1 was first cloned as an early estrogen-induced mRNA from guinea pig endometrial glandular epithelial cells (8). Its deduced amino acid sequences are completely conserved across the several species cloned to date, except the orangutan. GEC1 is widely distributed in mouse and human tissues (9–11). GEC1 is abundant in the central nervous system and is expressed throughout the rat brain (11, 12). Two other names have been used for GEC1: $GABA_A$ receptor-associated protein like 1 (GABARAPL1) (9) and Apg8L (12).

GEC1 belongs to the family of microtubule-associated proteins (MAPs). Other members of this family include $GABA_A$ receptor-associated protein (GABARAP) (13), Golgi-associated ATPase enhancer of 16 kDa (GATE16) (also named GABARAPL2) (14), and the yeast protein Atg8 (previously named apg8/aut7) (15). All four are 117-amino acid proteins. Light chain 3 of microtubule-associated protein 1 (MAP1-LC3) is a less similar member of the family (16). The identities of the amino acid sequence of GEC1 to its analogues are: GABARAP (86%), GATE16 (61%), Atg8 (55%), and LC3 (~30%).

Proteins of this family have been shown to play important roles in two biological functions: intracellular protein transport

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[2] The abbreviations used are: KOPR, κ opioid receptor; hKOPR, human KOPR; DOPR, δ opioid receptor; KCT, κ opioid receptor C-tail; DCT, δ opioid receptor C-tail; Atg8, yeast autophagy protein 8; GEC1, glandular epithelial cell 1, also named $GABA_A$ receptor-associated protein like 1, GABARAPL1; GABARAP, $GABA_A$ receptor-associated protein; GATE16, Golgi-associated ATPase enhancer of 16 KDa; LC3, light chain 3 of MAP 1A/1B; MAP, microtubule-associated protein; ANOVA, analysis of variance; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

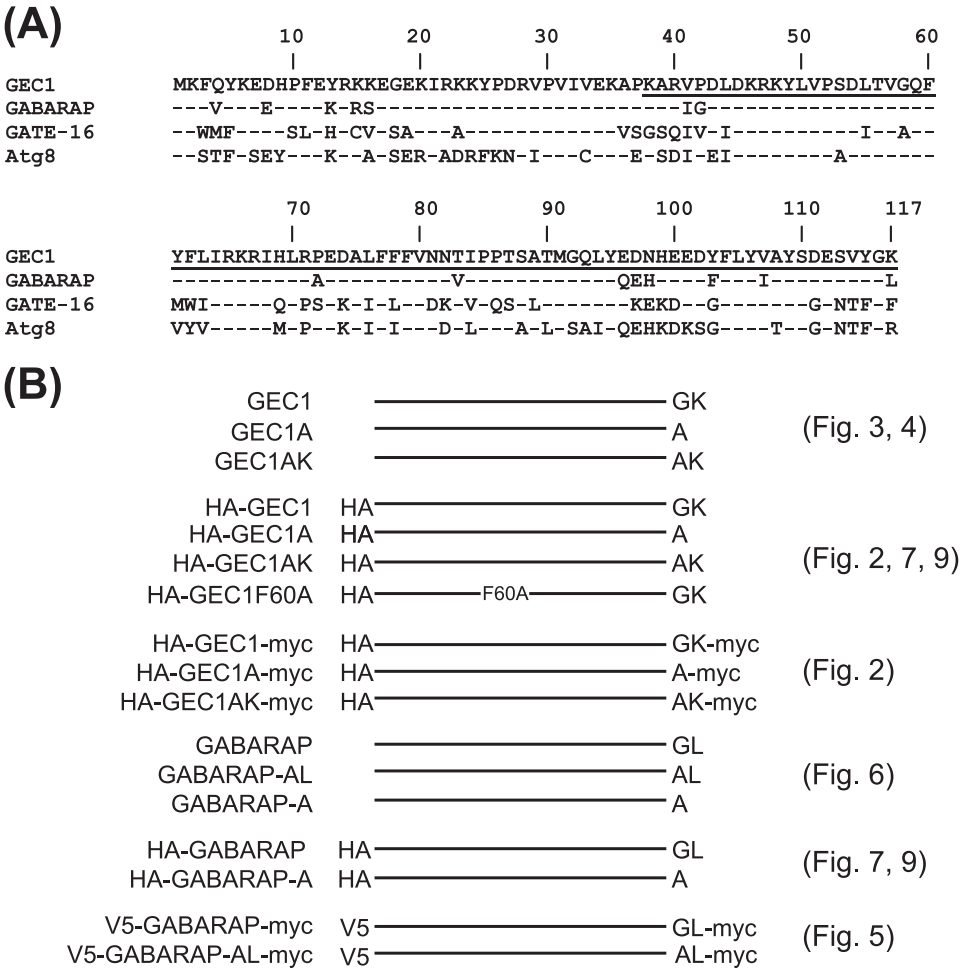

FIGURE 1. *A*, amino acid sequence comparison among GEC1 and its three analogues GABARAP, GATE16, and Atg8. The residue(s) C-terminal to the conserved glycine (equivalent to Gly^{116} of GEC1) of these analogues are cleaved, and the conserved glycine becomes the C terminus. Under starvation conditions, the glycine is conjugated to phosphatidylethanolamine. *B*, cDNA constructs used in this study. cDNA constructs of the mutants and wild types of GEC1 and GABARAP shown are inserted into the pcDNA3.1 or pCMV-HA mammalian expression vector.

and autophagy. GATE16, GABARAP, and GEC1 are involved in intracellular protein transport by enhancing vesicle fusion (7, 13, 14, 17–19). GATE16 is involved in intra-Golgi transport (14). GABARAP interacts with $GABA_A$ and AT1 angiotensin II receptors and transient receptor potential vanilloid 1 (TRPV1) and promotes their cell surface expression (13, 18, 20, 21). Atg8 and LC3 are essential for autophagy, and GABARAP and GATE16 are also shown to be involved. During autophagy, an evolutionarily highly conserved process occurring under nutrient deprivation conditions, cytoplasmic components and intracellular organelles are engulfed by autophagosomes (double membrane-bound compartments) and transported into lysosomes or vacuoles for degradation (22, 23). The process involves a series of biochemical reactions similar to ubiquitination (24, 25). The residues C-terminal to glycine (Gly^{116} of GABARAP, GATE16, or Atg8 and Gly^{120} of LC3) are cleaved in cytoplasm by Atg4 in yeast or its orthologue to yield Form I. Forms I are then modified sequentially by Atg7 (E1-like activating enzyme) and Atg3 (E2-like conjugating enzyme), resulting in covalent conjugations with phosphatidylethanolamine in membranes of autophagosomes, which are named Form II. Under conditions of starvation, Forms II of Atg8 and LC3 are enriched to promote autophagic activities (16, 24, 26). Currently, the Form II of LC3 is widely accepted as a marker of preautophagosomes and autophagosomes in mammalian cells (12, 27, 28). GEC1 was shown to undergo similar processes and associate with autophagic vesicles (12, 29, 44). Whether such C-terminal processing is needed for intracellular transport is unclear.

We demonstrated previously that both GEC1 and GABARAP promoted KOPR expression (6, 7). In this study, we investigated whether the C-terminal processing of GEC1 and GABARAP played a role in the observed enhancement in KOPR expression. We compared two GEC1 mutants and two GABARAP mutants with their wild-type counterparts, respectively, in biochemical processing and effects on hKOPR expression. For both proteins, one mutant has G116A substitution, and the other one has deletion of Lys^{117} in GEC1 and Leu^{117} in GABARAP besides G116A mutation (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials—[15, 16-^{3}H]-Diprenorphine (~56 Ci/mmol) was purchased from PerkinElmer Life Sciences. Naloxone was from Sigma-Aldrich. The following antibodies were used: rabbit anti-FLAG polyclonal antibody (F7425, Sigma-Aldrich); mouse monoclonal anti-HA (HA.11, Covance, Princeton, NJ); mouse

monoclonal anti-V5 (Invitrogen); mouse monoclonal anti-c-Myc (9E12, Santa Cruz Biotechnology, Santa Cruz, CA); and HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (Cell Signaling, Danvers, MA). Rabbit anti-GEC1 antibody (PA629p) was generated and purified as described in our previous publication (7, 11). SuperSignal West Pico Chemiluminescent and GelCode Blue stain reagents were from Pierce. Cell medium (DMEM/F-12, 1:1), Opti-MEM I reduced serum, fetal bovine serum (FBS), and Lipofectamine 2000 transfection reagent were acquired from Invitrogen. Small interfering RNAs (siRNAs) against mouse GEC1 and GABARAP genes (siGEC1 and siGABARAP, respectively) were purchased from Santa Cruz Biotechnology, and the negative siRNA control (siControl) was from Qiagen (catalog number 1027281, Valencia, CA). Glutathione *S*-transferase (GST) fusion system was from Novagen (GE Healthcare).

Cell Lines—A clonal CHO cell line stably expressing the FLAG-hKOPR was generated previously (30, 31), and the B_{max} value of FLAG-hKOPR was ~1.9 pmol/mg of protein (32). Two clonal Neuro 2A cell lines expressing FLAG-hKOPR or 3HA-hKOPR were similarly established expressing ~1 pmol/mg protein of hKOPR. All cells were cultured in 10-cm culture dishes or 6-well plates in DMEM/F-12 medium supplemented with 10% FBS, 0.2 mg/ml Geneticin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C.

Generation of cDNA Constructs—For C-terminal processing and receptor binding experiments, full-length GEC1 and GABARAP cDNAs and their mutants were inserted into the EcoRI/XhoI sites of the vector pcDNA3.1/Hygro(+) (Invitrogen). HA epitope was added 5′ to the initiation codon and c-Myc epitope immediately 5′ to the stop codon of the cDNAs in the same vector for expression of HA-tagged or HA- and -Myc-tagged proteins. V5-GABARAP-Myc and V5-GABARAP-AL-Myc were gifts from Dr. Richard W. Olson of UCLA School of Medicine (33). For immunofluorescence microscopy and protein pulldown assays, GEC1 and GABARAP and their mutants were inserted into SalI/XhoI sites of pCMV-HA vector (Clontech). hKOPR-EGFP was constructed into pLenti6/V5-TOPO vector (Invitrogen) and used as a regular CMV promoter-driven expression plasmid. Fig. 1 shows the cDNA constructs used in this study. For GST fusion proteins, KOPR-C-tail (Asp^{334}-Val^{380}) (KCT), DOPR-C-tail (Asp^{322}-Ala^{372}) (DCT), HA epitope-tagged GEC1 (HA-GEC1), and GABARAP (HA-GABARAP) were inserted into BamHI/XhoI site of pGEX-4T-1 vector.

Transient Transfection of GEC1, GABARAP, Their Mutants, and siRNAs—Lipofectamine-mediated DNA transfection experiments were performed by following the manufacturer's protocol with some modifications. Twenty-four hours before transfection, 1.8–2.0 million CHO-FLAG-hKOPR cells were seeded on each 10-cm cell culture dish. Transfection was carried out with 30 μl of Lipofectamine 2000, 10 μg of the cDNA constructs or the blank plasmid vector (control), and 6 ml of Opti-MEM medium per 10-cm dish. At 16 h after transfection, medium was replaced by 10 ml of Opti-MEM. Thirty hours following transfection, cells were harvested for receptor binding and Western blot experiments. GEC1 and GABARAP knockdown experiments were performed with Neuro 2A cells expressing 3HA-hKOPR similarly as above except that siGEC1, siGABARAP, and siControl were used. For immunofluorescence microscopy, HEK293 cells were cultured on coverslips placed in 12-well plates at 2×10^5 cells/well for 24 h. Cells were then co-transfected with 50 ng of hKOPR-EGFP (hκ-EGFP) and 25 ng of HA-tagged constructs as indicated in Fig. 8 with Lipofectamine 2000. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde in PBS buffer for 15 min. Immunofluorescence was performed by incubating cells with both mouse anti-HA (1/1000) and rabbit anti-giantin (1/1000) antibodies overnight at 4 °C and then with the secondary antibodies Texas Red-conjugated anti-mouse IgG (1/1000) and Alexa Fluor-350-conjugated anti-rabbit IgG (1/1000) at room temperature for 1 h. Images were acquired using a Nikon TE300 fluorescence microscope and a 60× oil objective lens and a Magnifire digital camera. The National Institutes of Health Image program and Adobe Photoshop were used for imaging processing. Care has been taken to avoid overexpression of the HA-GEC1 and its analogues. Transfection of 25 ng each of HA-GEC1 and its analogues was determined empirically so that the HA-GEC1 expressed at a level matched that of endogenous GEC1 (data not shown).

[^{3}H]Diprenorphine Binding to hKOPR in Intact Cells—Intact cell binding was performed as described previously (7). Briefly, 100,000 or 200,000 cells/tube were incubated with 1 nM [^{3}H]diprenorphine in PBS buffer for 1 h. Naloxone (10 μM) was used to define the nonspecific binding for total receptors, whereas dynorphin A (1–17) (1 μM) was used to define nonspecific binding for cell surface receptors. The KaleidaGraph program (Synergy Software) was used for data processing.

Protein Pulldown Assay—GST-KCT, GST-DCT, GST-HA-GEC1, and GST-HA-GABARAP proteins were prepared and bound to glutathione-Sepharose 4B beads as described previously (7). GST-HA-GEC1 and GST-HA-GABARAP bound to glutathione-Sepharose 4B beads were treated with biotinylated thrombin to produce HA-GEC1 and HA-GABARAP. After thrombin was removed by use of streptavidin-agarose, the purities of HA-GEC1 and HA-GABARAP were determined to be >90% by SDS-PAGE and GelCode Blue staining. The protein concentrations of purified HA-GEC1 and HA-GABARAP were determined by BCA reagent. DOPR-C-tail was chosen as negative control against KOPR-C-tail for their similarity in size and pI value (51 amino acids/47 amino acids and pI 9.21/pI 9.66). The GST-DCT also provides better separation in SDS-PAGE than GST from HA-GEC1 and HA-GABARAP, which is critical for reliable quantitation of bound GEC1 or GABARAP. Four concentrations of HA-GEC1 or HA-GABARAP (0.5 ml of 10, 20, 40, and 80 μg/ml) and 10 μl (20 μg of proteins) of Sepharose 4B-GST-KCT or -GST-DCT in TBS-T++ buffer (20 mM Tris, pH 7.4, 0.2 M NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.1% Tween 20) were incubated at 4 °C overnight on a rotating rack. An aliquot (5 μl) was taken from each concentration of HA-GEC1 or HA-GABARAP, diluted 50-fold in 2× Laemmli sample buffer, and used as the loading control in immunoblotting (see below). The incubation mixtures were washed 5 × 5 min with precooled TBS-T++ buffer by centrifugation and resuspension, and the buffer was aspirated. Thirty μl of 2× Laemmli sample buffer was then added to gel beads to dissociate bound

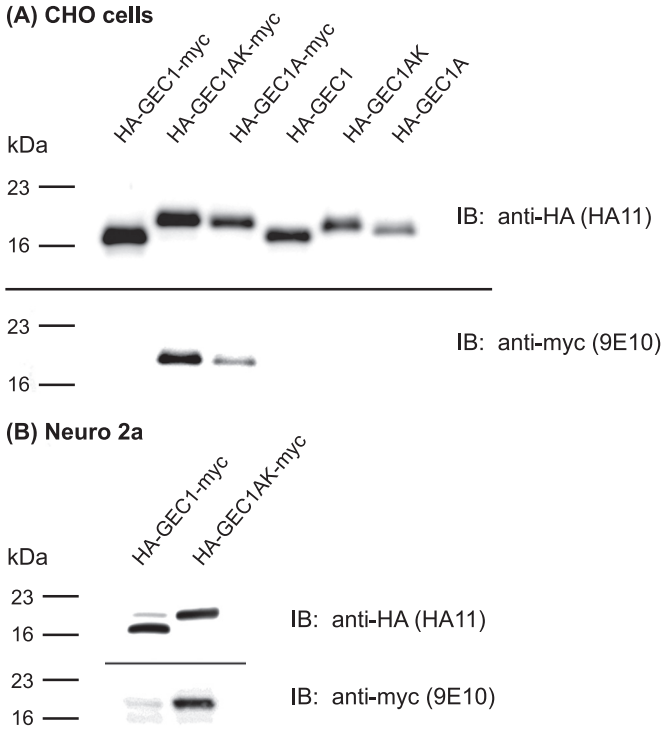

FIGURE 2. *A*, GEC1 undergoes C-terminal cleavage, but GEC1AK and GEC1A do not. Each construct was transfected into CHO cells with Lipofectamine. Forty hours later, cells were collected, dissolved in SDS loading buffer, and loaded onto 12% SDS-PAGE (4×10^5 cells/lane), and immunoblotting (*IB*) was performed with the antibodies indicated. *B*, HA-GEC1-Myc and the HA-GEC1AK-Myc were transfected into Neuro 2A cells and immunoblotted in a similar manner as in *A*. Note that C-terminal cleavage of HA-GEC1-Myc was incomplete, suggesting less robust Atg4B activity in Neuro 2A cells. The figures shown are the results of one of three independent experiments.

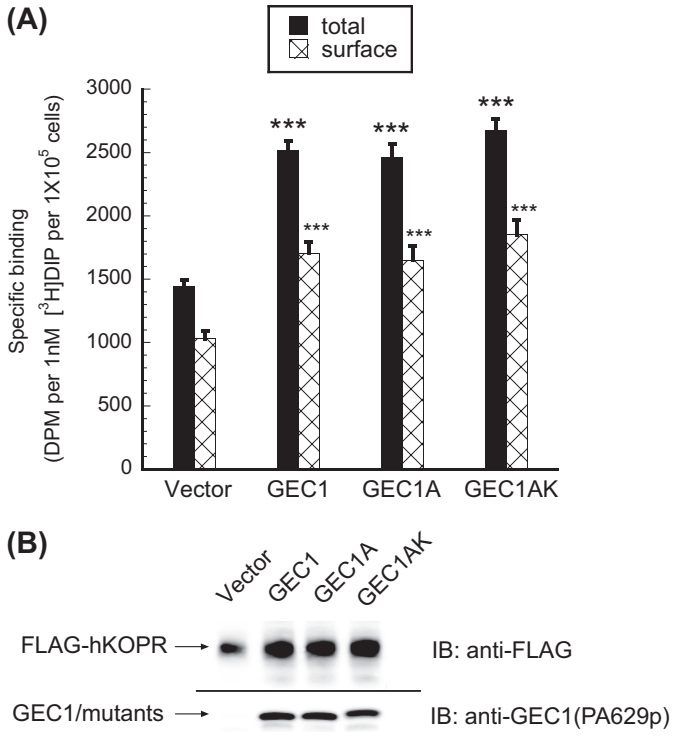

FIGURE 3. **C-terminal cleavage is not required for GEC1 to enhance total and cell surface KOPR expression.** CHO cells stably expressing FLAG-hKOPR were transfected as described in the legend for Fig. 2, and cells were harvested 40 h later. *A*, KOPR binding was performed with 1 nM [^{3}H]diprenorphine (*[^{3}H]DIP*) on intact cells using 10 μM Naloxone and 1 μM dynorphin to define nonspecific binding for total and cell surface receptors, respectively. The results are presented as mean ± S.E. of five experiments. ***, $p < 0.0005$ when compared with the vector group by one-way ANOVA followed by Dunnett's post hoc test. *B*, aliquots of each transfected cells from *A* were immunoblotted (*IB*) with the antibodies indicated. Each lane was loaded with 20 μg of proteins. The blot represents one of the four experiments performed.

proteins. The samples were heated at 60 °C for 10 min, and then 20 μl of each was loaded onto SDS-PAGE. Immunoblotting was performed with the anti-HA mAb (1:10,000), and the protein bands were quantified with the OptiQuant program as described previously (7). The linear relationship between intensities (digital line unit) and the amounts of HA-GEC1/HA-GABARAP was determined empirically to be within 5–80 ng (see Fig. 9*B*), and the incubation concentrations were adjusted so that the intensities of bound proteins were within this linear range. Ten, 20, 40, and 80 ng of HA-GEC1 and HA-GABARAP were included in each immunoblotting as the loading controls and were also used to calculate bound proteins in the same data set (see Fig. 9, *B* and *C*).

SDS-PAGE and Immunoblotting—Cells were harvested using Versene buffer, solubilized in 2× Laemmli sample buffer, and subjected to Tricine-SDS-PAGE in 10 or 12% separating gel as described previously (6, 7). The separated protein bands were transferred to Immobilon-P polyvinylidene difluoride membranes on which immunoblotting was carried out with the primary antibodies indicated in the figure legends, horseradish peroxidase-linked secondary antibody, and SuperSignal West Pico chemiluminescent reagents. Antibodies were used at the following dilutions: rabbit anti-FLAG polyclonal antibody, 1:2000; mouse monoclonal anti-HA, 1:4000 unless indicated otherwise; mouse monoclonal anti-V5, 1:5000; mouse monoclonal anti-c-Myc, 1:2000; horseradish peroxidase-linked secondary antibodies, 1:10,000. The protein bands were visualized and digitalized with the Fuji LAS-1000 Plus gel documentation system (Fuji Film, Tokyo, Japan).

RESULTS

Role of the Gly^{116} in the C-terminal Processing of GEC1—When HA-GEC1-Myc, HA-GEC1AK-Myc, or HA-GEC1A-Myc was transiently transfected into CHO-FLAG-hKOPR cells, all the expressed proteins were recognized by antibody against the HA epitope (Fig. 2*A*, *upper panel*). Although HA-GEC1-Myc, HA-GEC1AK-Myc, or HA-GEC1A-Myc had almost identical calculated molecular mass, HA-GEC1-Myc migrated faster than the other two, yielding similar relative molecular weight (M_r) as HA-GEC1. However, HA-GEC1AK-Myc and HA-GEC1A-Myc had higher M_r value than HA-GEC1AK and HA-GEC1A, respectively. In addition, HA-GEC1AK-Myc and HA-GEC1A-Myc, but not HA-GEC1-Myc, were recognized by anti-c-Myc antibody (Fig. 2*A*, *lower panel*). These findings indicate loss of c-Myc from HA-GEC1-Myc due to cleavage at the C terminus of GEC1, but not from HA-GEC1AK-Myc and HA-GEC1A-Myc. Thus, C-terminal modifications do occur to GEC1, perhaps similar to Atg8, for which the Gly^{116} is required (24, 25). Similar results were also obtained in blank Neuro2A cells (Fig. 2*B*), indicating that the cleavage at the C terminus of GEC1 does not depend on cell types or KOPR expression.

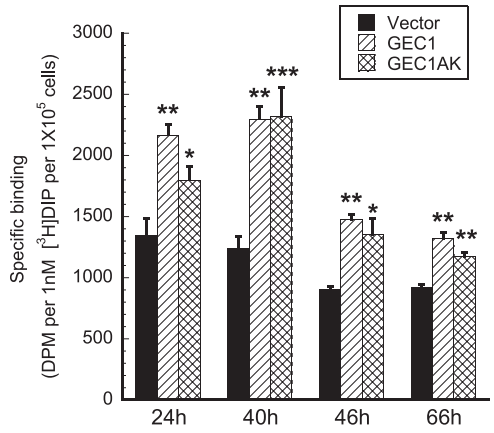

FIGURE 4. **GEC1 and GEC1AK affect KOPR expression with similar time courses.** Transfection and intact cell binding were performed as described in the legend for Fig. 3. KOPR binding was performed with ~1 nM [^{3}H]diprenorphine (*[^{3}H]DIP*) on intact cells using 10 μM Naloxone to define nonspecific binding. Each value is mean ± S.E. of three or four experiments. ***, $p < 0.0005$, **, $p < 0.005$, *, $p < 0.05$ when compared with the vector control group at same time point by one-way ANOVA followed by Dunnett's post hoc test.

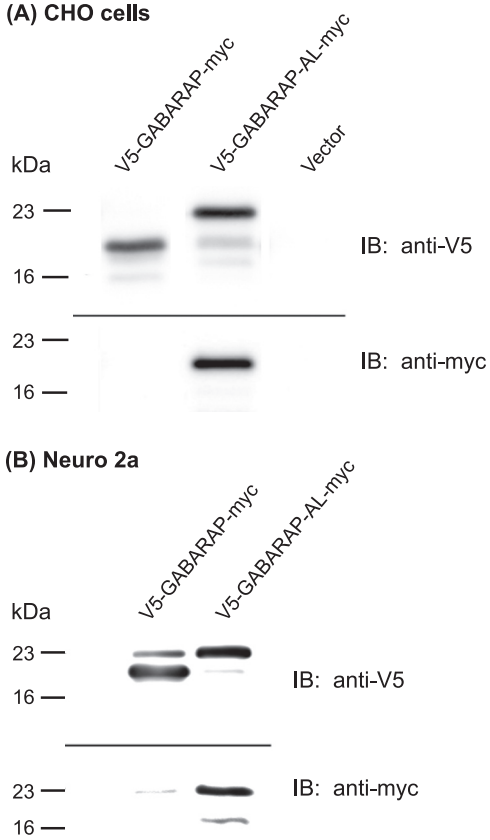

FIGURE 5. **The C terminus of the wild-type GABARAP, but not the G116A mutant (GABARAP-AL), is cleaved, similar to GEC1, in CHO cells (*A*) and in Neuro 2A cells (*B*).** The experiment was performed similarly as described in the legend for Fig. 2. The blots represent one of the three experiments. *IB*, immunoblot.

Effect of G116A Mutation on GEC1-induced Increase of hKOPR Expression—Following transient transfection, GEC1A and GEC1AK enhanced total and cell surface hKOPR expression to similar extents as the wild-type GEC1, as determined by both radioligand binding and immunoblotting (Fig. 3). Thus,

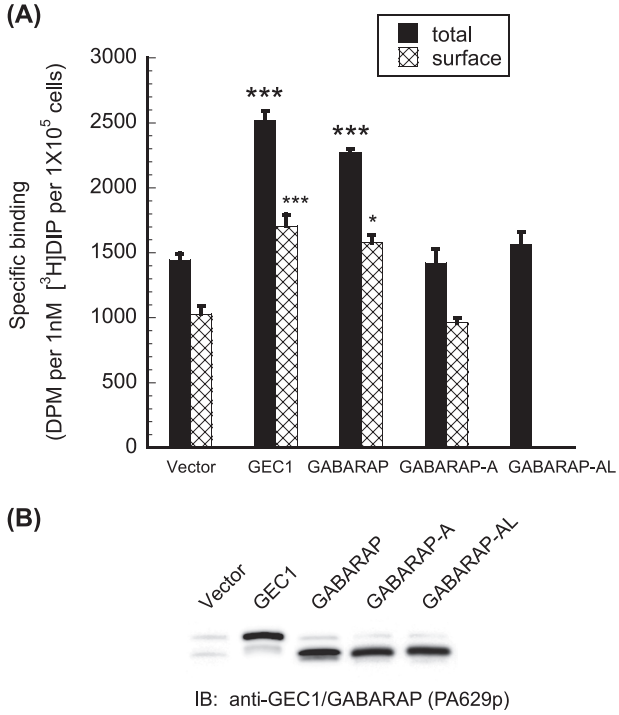

FIGURE 6. **Contrary to GEC1, the C-terminal cleavage is essential for GABARAP to enhance KOPR total and cell surface expression.** Experiments were carried out as described in the legend for Fig. 3. *A*, each value is mean ± S.E. of four experiments. *[^{3}H]DIP*, [^{3}H]diprenorphine. *B*, the immunoblot (*IB*) represents one of the three experiments. *, $p < 0.05$, ***, $p < 0.0005$ when compared with the vector control by one-way ANOVA followed by Dunnett's post hoc test.

C-terminal modification is not required for GEC1 to enhance expression of the hKOPR. In addition, when compared with the vector control, GEC1, GEC1A, and GEC1AK did not change the ratio of cell surface to total receptors, indicating that all three proteins did not act differentially on intracellular or cell surface receptors and that both mutants act in similar manners as the wild type. Moreover, GEC1AK enhanced hKOPR expression with a time course similar to that of GEC1 (Fig. 4).

These results are different from those of Chen *et al.* (33) that C-terminal modification is required for GABARAP to enhance cell surface expression of the γ subunit of the $GABA_A$ receptor. We have shown previously that GABARAP interacts with the KOPR and enhances KOPR levels, but to a lesser extent than GEC1 (6). We, therefore, examined whether C-terminal modification was required for GABARAP to enhance KOPR expression.

Role of Gly^{116} in the C-terminal Processing of GABARAP—Two GABARAP constructs, V5-GABARAP-Myc and V5-GABARAP-AL-Myc (G116A mutant), were transfected into CHO cells (Fig. 5*A*) or Neuro 2A cells (Fig. 5*B*), and immunoblotting was performed with antibodies against V5 and c-Myc. Although anti-V5 antibody recognized both proteins (Fig. 5, *A* and *B*, *upper panel*), anti-c-Myc antibody only recognized the G116A mutant (Fig. 5, *A* and *B*, *lower panel*). In addition, V5-GABARAP-Myc yielded lower M_r than V5-GABARAP-AL-Myc (Fig. 5, *A* and *B*, *upper panel*). These results demonstrate that similar to GEC1, the C terminus of the wild-type GABARAP, but not the G116A mutant, is cleaved and that the Gly^{116} of GABARAP is required for its C-terminal processing.

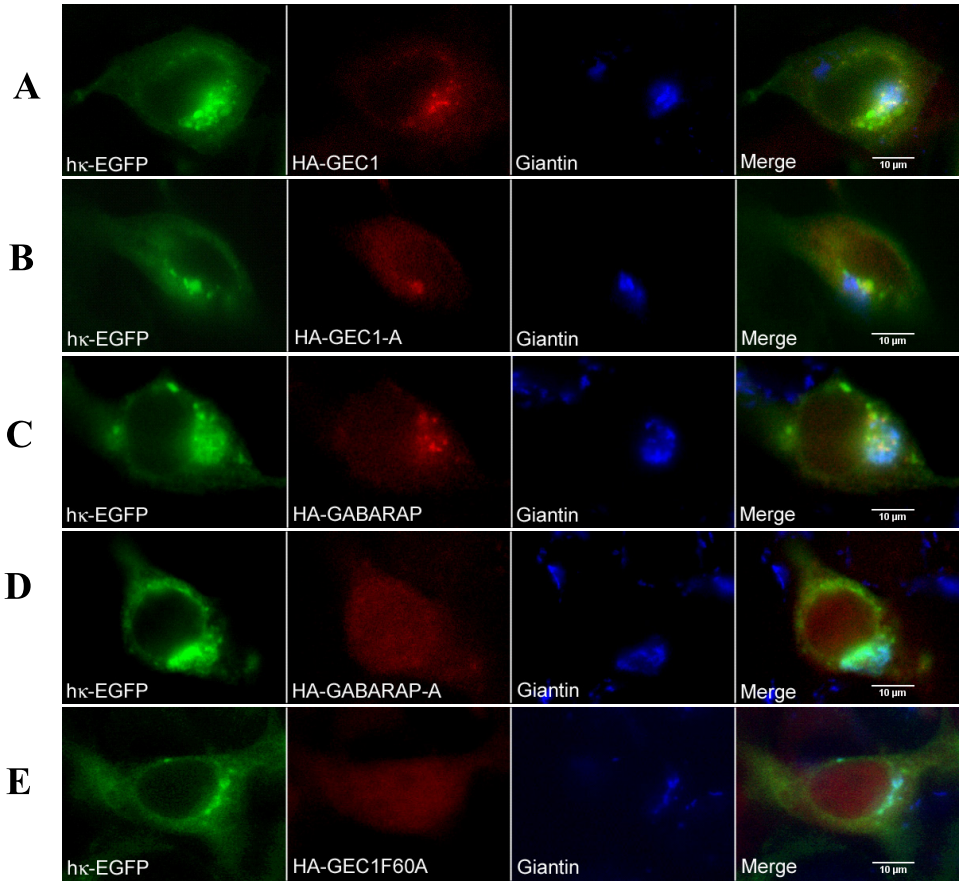

FIGURE 7. **Co-localization of KOPR with GEC1, GEC1-A, and GABARAP, but not with GABARAP-A and GEC1F60A.** HEK293 cells were cultured on coverslips for 24 h and then co-transfected with KOPR-EGFP (hκ-EGFP) and HA-tagged constructs as indicated with Lipofectamine 2000. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde. Immunofluorescence was performed with mouse anti-HA antibodies followed by Texas Red-conjugated anti-mouse IgG for wild-type and mutant GEC1/GABARAP (*red*) and rabbit anti-giantin antibody and then Alexa Fluor-350-conjugated anti-rabbit IgG for the Golgi marker giantin (*blue*). Images shown are KOPR-EGFP (*green*, *first column*) co-expressed with HA-GEC1 (*A*), HA-GEC1-A (*B*), HA-GABARAP (*C*), HA-GABARAP-A (*D*), and HA-GEC1F60A (*E*) (*red*, *second column*). The Golgi marker giantin is shown in the *third column* (*blue*). Merged images are shown in the *fourth column*. These images are representatives of at least 50 images per row from four independent experiments.

Effect of G116A Mutation on GABARAP-induced hKOPR Expression—GABARAP, GABARAP-AL (G116A mutant), and GABARAP-A (containing G116A mutation and Leu^{117} deletion) were examined along with GEC1 for their effects on expression of hKOPR (Fig. 6). GABARAP transfection increased both total and cell surface hKOPR expression by more than 80% without changing the percentage of total receptors on the cell surface. In contrast, GABARAP-AL and GABARAP-A failed to show any increase. These results demonstrate that, unlike GEC1, GABARAP requires C-terminal cleavage to enhance hKOPR expression. GABARAP did not alter the percentage of total receptors on the cell surface, similar to GEC1. The degree of hKOPR increase induced by GABARAP was lower than that of GEC1, consistent with our previous findings (6).

Distribution of GEC1, GABARAP, and Their Mutants in Cells and Relationship to hKOPR Distribution—We used HEK293 cells for localization studies instead of CHO cells because CHO cells have much larger nuclei and very limited cytosol space, making it difficult to visualize subcellular organelles. Cells were co-transfected transiently with KOPR-EGFP (*green*) and HA-tagged GEC1, GEC1A, GEC1-F60A GABARAP, or GABARAP-A. We showed previously that GEC1-F60A did not interact with hKOPR and did not enhance hKOPR expression (6). Immunofluorescence was performed (Fig. 7) for HA-GEC1 and analogues with anti-HA antibodies (*red*) and giantin with giantin antibodies (*blue*), a marker for the Golgi apparatus. Staining of HA-GEC1, HA-GEC1A, and HA-GABARAP was punctate in cells and co-localized with intracellular KOPR-EGFP and giantin. In contrast, HA-GABARAP-A and HA-GEC1F60A had a diffused pattern and did not show significant co-localization with KOPR-EGFP or giantin. These results suggest that the localization of GEC1 and GABARAP with KOPR is required for them to enhance receptor expression.

It should be noted that cells transiently transfected with KOPR-EGFP were used for immunofluorescence studies, whereas stably transfected cells were used for the studies in which KOPR quantitation was involved. The reason for these choices is that transiently transfected cells have a much larger intracellular pool of receptor precursors than stably transfected cells, which is especially true when examined within 24 h after transfection (the condition used in experiments for Fig. 7). The high levels of intracellular receptor precursors facilitated studies on possible spatial differences displayed by GEC1,

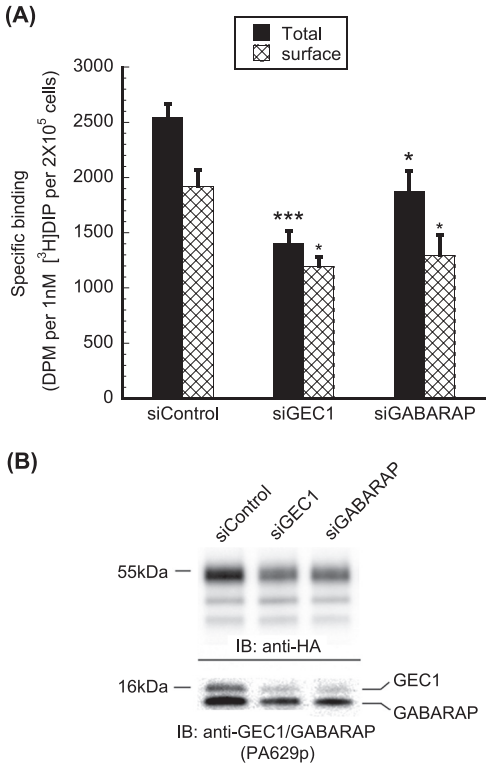

FIGURE 8. **Knockdown of endogenous GEC1 or GABARAP reduces both total and cell surface KOPR expression in Neuro 2A cells.** siRNAs targeting mouse genes *gec1* (siGEC1) or *gabarap* (siGABARAP) were transfected into Neuro 2A cells stably expressing 3HA-hKOPR. siControl, a non-targeting siRNA, was used as the negative control. Thirty hours after transfection, cells were collected for receptor binding and immunoblotting assays. *A*, total and cell surface receptor binding was conducted with 1 nM [^{3}H]diprenorphine (*[^{3}H]DIP*) using 10 μM Naloxone and 1 μM dynorphin to define nonspecific binding for total and cell surface receptors, respectively. The results are presented as mean ± S.E. of four independent experiments. ***, $p < 0.0005$, *, $p < 0.05$ when compared with siControl group by one-way ANOVA followed by Dunnett's post hoc test. *B*, aliquots of transfected cells from *A* were immunoblotted (*IB*) with the antibodies indicated. Each lane was loaded with 20 μg of proteins. The blot represents one of the three experiments performed.

GABARAP, and their mutants and their co-localization of the hKOPR in the Golgi (Fig. 7).

Effect of GEC1 and GABARAP Knockdown on hKOPR Expression—Both CHO cells and Neuro 2A cells express GEC1 and GABARAP endogenously, which were readily detectable by immunoblotting with the GEC1 antibody PA629p (Fig. 8*B*). We have shown previously that PA629p has significant cross-reactivity with GABARAP and that GABARAP has a lower relative molecular weight than GEC1 in SDS-PAGE (11). Because siRNAs of mouse, but not Chinese hamster, origin are readily available, Neuro 2A cells (mouse) expressing 3HA-hKOPR were used. Transfection of siGEC1 or siGABARAP reduced their targeting protein levels when compared with siControl (Fig. 8*B*, *bottom panel*). Importantly, knockdown of GEC1 or GABARAP decreased the total and cell surface hKOPR expression as shown by receptor binding (Fig. 8*A*) and immunoblot (Fig. 8*B*). Note that the GEC1 siRNA showed significant cross-reactivity to GABARAP and *vice versa*.

Interaction with KOPR-C-tail, Comparison between GEC1 and GABARAP—Pulldown techniques were employed for these experiments, using GST-KOPR-C-tail or GST-DOPR-C-tail (as the control) coupled to glutathione-Sepharose and HA-GEC1 and HA-GABARAP purified from GST fusion products. This combination allowed reproducible results. As shown in Fig. 9, at 40 and 80 μg/ml, HA-GEC1 had significantly more binding to GST-KCT than HA-GABARAP. In contrast, DOPR-C-tail did not bind HA-GEC1 or HA-GABARAP.

It should be noted that the binding has not reached saturation because of technical limitations. To reach saturation, the amount of GST-KOPR-C-tail or -DOPR-C-tail beads has to be very low, and the amount we used was at the limit of reproducible pipetting. In addition, the staining density of HA-GEC1 or HA-GABARAP has to be within the linear detection range. Therefore, instead of a "saturation curve," here we presented data from low "ligand" concentrations. The results, nevertheless, revealed that GEC1 binds to KOPR-C-tail at higher affinity than GABARAP (Fig. 9*C*).

DISCUSSION

In this study, we found that C-terminal cleavage of GEC1 is not required for its effects in enhancing expression of the hKOPR; however, GABARAP requires C-terminal cleavage to have the same effect. In addition, we demonstrated that GEC1 had stronger interaction with KOPR-C-tail than GABARAP, which may account for the observed difference.

Similarities and Differences between GEC1 and GABARAP—The C termini of GEC1 and GABARAP were cleaved, and the G116A mutation blocked this process, indicating that the Gly^{116} residue is critical for C-terminal processing. Our results are in agreement with the observations of Tanida *et al.* (27, 29) and Chen *et al.* (33) and support the notion that the C-terminal processing is universal for all the GEC1 analogues identified to date. In addition, we found that GEC1, GEC1A, as well as GEC1AK were equally effective in promoting KOPR expression. In contrast, GABARAP increased KOPR expression, whereas GABARAP-A and GABARAP-AL did not. Therefore, the C-terminal processing is not necessary for GEC1 to enhance KOPR expression but is required for GABARAP to have the same effect. Our results on GABARAP are similar to those of Chen *et al.* (33) on the γ subunit of the $GABA_A$ receptor but different from those of Alam *et al.* (36) on angiotensin II type 1A receptor (see below).

The marked difference between GEC1 and GABARAP in their requirement of C-terminal cleavage for promoting KOPR expression was unexpected. C-terminal modification by the Atg4B-Atg7-Atg3 system defines a common biochemical pathway for this family of proteins and presumably leads to similar functional consequences. In addition to and/or independent of these shared features, there may be factors that set diverging points for members of the family. We demonstrated that both wild-type GEC1 and wild-type GABARAP enhanced KOPR expression with GABARAP having a lower degree of increases (Fig. 6) (also see Ref. 6), which may reflect lower affinities for the hKOPR. This notion is supported by the data from quantitative analysis of the pulldown assay that HA-GEC1 had significantly stronger association with the KOPR-C-tail than HA-GABARAP (Fig. 9*C*). In addition, using yeast two-hybrid technique, we showed previously that on quadruple dropout plates, the colony-forming units were 83 ± 10 and 66 ± 5 ($n = 3$, $p < 0.05$, Student's *t* test) for interaction of KOPR-C-tail with

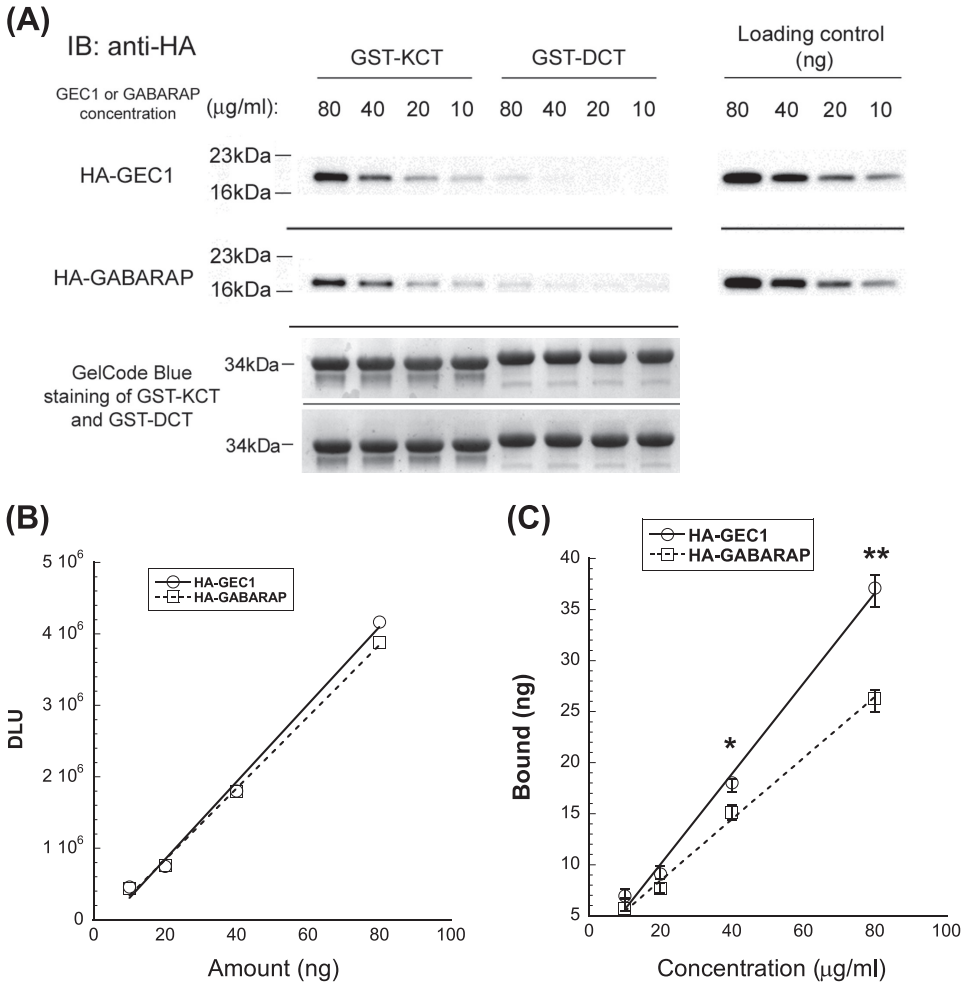

FIGURE 9. **GEC1 exhibits higher affinity for KOPR-C-tail than GABARAP.** Protein pulldown assays were performed to compare the interaction strength of the KOPR-C-tail with GEC1 and GABARAP. Purified GST-KOPR-C-tail (GST-KCT) or GST-DOPR-C-tail (GST-DCT) proteins were coupled to glutathione-Sepharose 4B beads. HA-GEC1 and HA-GABARAP were obtained by cleavage with thrombin of GST-HA-GEC1 and GST-HA-GABARAP coupled to glutathione-Sepharose 4B beads. Ten μl each of GST-KCT or GST-DCT beads was incubated overnight at 4 °C with different concentrations of HA-GEC1 or HA-GABARAP followed by immunoblotting analysis. *A*, one of the four immunoblotting (*IB*) results obtained. An aliquot of HA-GEC1 or HA-GABARAP from each concentration (10, 20, 40, and 80 μg/ml) was loaded as loading control (*upper right*) for every experiment and was used to generate standard curves as shown in *B*. *DLU*, digital line unit. *C*, the bound HA-GEC1 or HA-GABARAP was quantified using the standard curves in each data set and plotted against the incubation concentration. The result was presented as mean ± S.E. of four experiments. *, $p < 0.05$, **, $p < 0.01$ by one-way ANOVA followed by Dunnett's post hoc test.

GEC1(38–117) and GABARAP(38–117), respectively (7), indicating that GABARAP(38–117) had weaker binding to the KOPR C-tail than GEC1(38–117). Intact GEC1 or GABARAP did not work in yeast two-hybrid assays, most likely because of their strong association with microtubules via the N-terminal domains (6, 7).

Deletion of Gly^{116} and Lys^{117} did not show any effect on direct interaction of GEC1 with KOPR C-tail in yeast two-hybrid assay (6). In addition, based on our GEC1 model (6) and crystal structures of GABARAP (Protein Data Bank code 1GNU) (37–39), Gly^{116} is unlikely to contribute to any differences in their binding surface. Therefore, we postulate that their "intrinsic" affinities to KOPR are independent of the C-terminal processing.

We have demonstrated that the interaction between hKOPR C-tail and GEC1 is mediated by direct contacts between the kinked hydrophobic fragment in hKOPR C-tail (containing Phe^{345}, Pro^{346}, and Met^{350}) and the curved hydrophobic surface in GEC1 around the S2 β-strand formed by Tyr^{49}, Val^{51}, Leu^{55}, Thr^{56}, Val^{57}, Phe^{60}, and Ile^{64} (6). Despite the high amino acid sequence identity between GABARAP and GATE16, there are a few differences in their x-ray crystal structures (39, 40). The crystal structure of GEC1 has not been resolved; however, it is likely to be similar to that of GABARAP, with some subtle differences. These differences may contribute to lower affinity of GABARAP for the hKOPR C-tail and thus lower levels of enhancement in hKOPR expression.

We postulate that two independent factors may contribute to enhancing the effects of GEC1 and analogues on hKOPR cell surface expression: 1) the affinity of GEC1 and analogues for the hKOPR; and 2) C-terminal modifications, which facilitate association of GEC1 and analogues with intracellular membranes, where the hKOPR to be exported resides. If the affinity is sufficiently high, as in the case of GEC1, adequate amounts of GEC1 will interact with hKOPR even without C-terminal modifications and thus enhance hKOPR expression. If the affinity for the hKOPR C-tail is not high enough, as in the case of GABARAP, enhanced membrane association with C-terminal modifica-

tions is needed to have full effects. Our immunofluorescence results (Fig. 7) support this notion. HA-GEC1, HA-GABARAP, and their G116A mutants co-transfected with KOPR-EGFP showed remarkably different staining patterns. HA-GEC1, HA-GABARAP, and HA-GEC1A had punctate staining in the cytoplasmic space, which co-localized significantly with KOPR-EGFP in the Golgi apparatus, whereas GABARAP-A had diffuse distribution, with little co-localization with KOPR-EGFP. In addition, the GEC1F60A mutant, which had substantially lower binding to the KOPR C-tail and had no enhancing effect on KOPR expression (6), showed diffuse staining and a low level of co-localization with KOPR although this mutant most likely undergoes C-terminal modification. It is conceivable that higher intrinsic affinity of GEC1 to KOPR alone allows GEC1A to be recruited sufficiently to Golgi by KOPR, where it enhances transport. In contrast, without the benefit of membrane association via phosphatidylethanolamine conjugation, GABARAP-A fails to be recruited sufficiently by KOPR due to its lower binding affinity to the receptor. Whether this inference is valid remains to be examined.

Recent studies on the effects of GABARAP on trafficking of different receptors have yielded different results. Chen *et al.* (33) have shown that cleavage at the C terminus in GABARAP is required for GABARAP-mediated $GABA_A$ trafficking and enhanced GABA-induced currents. Alam *et al.* (36), however, demonstrated that the wild-type GABARAP and its G116A mutant were equally effective in stimulating surface expression and signaling activity of angiotensin II type 1A receptor. The two findings may seem contradictory to each other but can be explained by the different affinities of GABARAP for the γ2 subunit of the $GABA_A$ receptor and for the angiotensin II type 1A receptor. In addition, C-terminal modification of GABARAP is required for enhancing surface expression of the KOPR, but not angiotensin II type 1A receptor, suggesting that the interactions of GABARAP and its analogues with receptors are related to their affinity for the receptor, more specifically the fragment of the receptor, but independent of whether the receptor is coupled to G proteins or not.

Redundant Functions of GEC1 and GABARAP—Both GEC1 and GABARAP have been shown to bind to membrane-bound proteins. GEC1 has been shown to interact with $GABA_A$ receptor (35), KOPR (7), and AT1 angiotensin II receptor (20) and enhance cell surface expression of the KOPR (7). GABARAP is associated with and increases cell surface expression of $GABA_A$ receptor (13, 18), KOPR (6), Na^+-dependent P_i cotransporter, sodium P_i IIa (41), AT1 angiotensin II receptor (20), and TRPV1 (21). In addition, GABARAP also binds transferrin receptor (42). Because of the high degree of identity, GABARAP and GEC1 are likely to have overlapping functions. The findings that $GABA_A$ receptor, KOPR, and AT1 angiotensin II receptor interact with both GEC1 and GABARAP (6, 13, 20, 35) support this notion. In GABARAP-null mice, there is no change in $GABA_A$ receptor levels or membrane clustering (43), most likely due to the redundant functions between the two proteins.

Possible Molecular Mechanisms of GEC1- or GABARAP-promoted Trafficking—We have shown that GEC1 facilitates transport of the KOPR from endoplasmic reticulum to Golgi to plasma membranes. However, the molecular mechanisms of this facilitation are not clear. Both GEC1 and GABARAP bind tubulin and *N*-ethylmaleimide-sensitive factor (6, 7, 17, 34, 35); therefore, their functions are related to transport along microtubules. Because they are not motor proteins like the anterograde transport protein kinesin, they do not move vesicles actively along microtubules. Cook *et al.* (20) postulated that GABARAP binds microtubules and vesicular cargo such as the AT1 angiotensin II receptor and, by doing so, stabilizes the kinesin-vesicle complex on microtubules as kinesin moves the cargo forward. In addition, the GABARAP binding stabilizes vesicle cargo on microtubules after one kinesin molecule dissociates from microtubules and before another one associates. It is conceivable that GEC1 acts in a similar manner to facilitate anterograde transport of the KOPR, which remains to be studied.

Conclusion—Our finding that C-terminal modifications are not required for GEC1 to enhance KOPR expression indicates that this function is independent of the involvement of GEC1 in autophagy and clearly demonstrates that this family of proteins has two very distinct functions.

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