Role of C-terminal Membrane-proximal Basic Residues in Cell Surface Trafficking of HIV Coreceptor GPR15 Protein

Yukari Okamoto, Joshua David Bernstein, and Sojin Shikano

From the Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607

Received for publication, December 17, 2012, and in revised form, February 5, 2013 Published, JBC Papers in Press, February 19, 2013, DOI 10.1074/jbc.M112.445817

Cell surface density of G protein-coupled receptors (GPCRs) is controlled by dynamic molecular interactions that often involve recognition of the distinct sequence signals on the cargo receptors. We reported previously that the RHR-type dibasic motif in the distal C-terminal tail of an HIV coreceptor GPR15 negatively regulates the cell surface expression by mediating the coatomer protein I complex-dependent retrograde transport to the endoplasmic reticulum (ER). Here we demonstrate that another pair of basic residues (Arg310-Arg311) in the membrane-proximal region of the C-terminal tail plays a pivotal role in mediating the anterograde trafficking of GPR15. The Ala mutation of the C-terminal membrane-proximal basic residues (MPBRs) (R310/311A) abolished the O-glycosylation and cell surface expression of GPR15. The subcellular fractionation and immunocytochemistry assays indicated that the R310/311A mutant was more localized in the ER but much less in the trans-Golgi when compared with the wild-type GPR15, suggesting the positive role of Arg310-Arg311 in the ER-to-Golgi transport of GPR15. Sequence analysis on human GPCRs showed that the basic residues are frequent in the membrane-proximal region of the C-terminal tail. Similar to GPR15, mutation of the C-terminal MPBRs resulted in a marked reduction of the cell surface expression in multiple different GPCRs. Our results suggest that the C-terminal MPBRs are critically involved in mediating the anterograde trafficking of a broad range of membrane proteins, including GPCRs.

Background: The role of membrane-proximal basic residues (MPBRs) in protein trafficking is not well understood.

Results: Mutation of C-terminal MPBRs reduced cell surface expression of GPR15 and other GPCRs.

Conclusion: C-terminal MPBRs are necessary for cell surface trafficking of GPCRs, including GPR15.

Significance: C-terminal MPBRs play a pivotal role in cell surface trafficking of GPCRs.

GPCRs represent the largest class of cell surface receptors and play a central role in regulating a plethora of biological pathways. The magnitude of GPCR-mediated signaling depends largely on their cell surface density. Thus, elucidation of molecular mechanisms underlying the cell surface delivery of GPCRs will provide an important basis for therapeutic intervention as well as for understanding GPCR biology (1). However, compared with the extensive studies made on ligand-induced endocytic pathways (2–5), the mechanisms governing the anterograde trafficking of GPCRs to the cell surface are less well understood.

The export from the ER is the first critical step in membrane protein trafficking. ER export is facilitated by the cargo selection process that involves recognition of distinct sequence signals on the cargo proteins by ER export machineries such as the coatomer protein (COP) II complex (6–9). Various sequence motifs have been found to mediate ER export of membrane protein cargos by directly interacting with COPII components (10). One of the less well characterized signals among them is the basic residues located proximal to the transmembrane (TM) segment. Membrane-proximal basic residues (MPBRs) have been reported to be involved in anterograde trafficking in a limited number of proteins. The basic domain in the C-terminal tail of chemokine receptor CCR5 was found to support optimal surface expression by cooperating with the adjacent downstream Cys cluster (11). The C-terminal MPBRs were also required for both cell surface expression and signaling function of melanin-concentrating hormone receptor 1 (MCH1R) (12). The positive role of MPBRs in anterograde trafficking was also reported for the Golgi-resident glycosyltransferases that employ type II membrane protein topology with a short N-terminal tail in the cytoplasmic side (13). In this case, specific mutations of these MPBRs abolished the interaction of the enzyme with Sar1 GTPase and blocked the export of enzymes from the ER. A more recent study in the α-2B adrenergic receptor (ADRA2B) showed that the MPBRs in the third cytoplasmic loop are necessary for receptor interaction with COPII protein Sec24D (14).

We have reported previously that GPR15, an orphan GPCR that serves as an alternative coreceptor for HIV and simian immunodeficiency virus, requires the phosphorylation-dependent binding of 14-3-3 proteins to the distal C terminus for its optimal cell surface expression (15). The 14-3-3 binding partially interferes with the access of COPI to the RHR-type dibasic ER localization motif that is adjacent to the C-terminal 14-3-3 binding site and thereby releases the receptor from the Golgi-to-ER retrograde transport pathway. GPR15 carries another...
Membrane-proximal Basic Residues in GPCR Trafficking

dibasic sequence (Arg310-Arg311) in the membrane-proximal region of the C-terminal tail. We were interested in whether these MPBRs are also involved in the cell surface trafficking of GPR15.

MPBRs have been known for their role in governing protein topology (16–18). Orientation of the nascent polypeptides during ER insertion is dictated by the charged residues flanking the hydrophobic core, where the more positive end is predominantly positioned to the cytoplasmic side of the membrane (“positive-inside rule”). Indeed, our sequence analyses of the human membrane protein database demonstrated the prevalence of basic residues in the membrane proximal region of the C-terminal tail in GPCRs and other receptor proteins. However, our studies using Ala mutant GPR15 revealed that these MPBRs were not necessary for maintaining the protein topology but instead were critically required for ER-to-Golgi export and cell surface expression of GPR15. We further show that multiple different human GPCRs do require C-terminal MPBRs for their surface expression, similar to GPR15. Together, our results suggest that C-terminal MPBRs play an important role in mediating the anterograde trafficking of a broad range of membrane proteins, including GPCRs.

EXPERIMENTAL PROCEDURES

Plasmids—The human GPR15 gene was cloned in the pCMV vector with N-terminal HA or Myc epitope tag as described previously (15). The GPR15 without tag was cloned in the pCDNA3.1(+) vector (Invitrogen). The entire C-terminal cytoplasmic tail (amino acids 306–360) of GPR15 with or without Ala mutations in Arg310–Arg311 was cloned in the pCMVGST vector to generate GST fusions. HA-tagged hamster Sar1b WT and the H79G mutant in the pTARGET vector were obtained from Dr. J. Lippincott-Schwartz (National Institute of Health), and the H79G mutant in the pET-11d vector were obtained from Dr. W. Balch (Scripps Research Institute). The human Sec23a and Sec24d genes, obtained from the DNASU Plasmid Repository (Tempe, AZ), were cloned in the pCMVmyc and pCMVGFP vectors. The human cDNAs for ADRA2B (α2-B adrenergic receptor), ADRA2C (α2-C adrenergic receptor), CCR4 (C-C chemokine receptor type 4), CXCR3 (C-X-C chemokine receptor type 3), FFA2 (free fatty acid receptor 2), GPR39 (G protein-coupled receptor 39), NPFFR1 (neuropeptide FF receptor 1), and SSTR4 (somatostatin receptor type 4) were obtained from DNA Su and cloned in the pCMVmyc vector. Site-directed mutagenesis was performed by overlap extension PCR.

Antibodies (Abs)—The following Abs were used: mouse HA, rabbit 14-3-3β, and mouse tubulin from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA); rabbit HA from Cell Signaling Technology, Inc. (Danvers, MA); mouse Myc (both non-conjugated and Alexa Fluor 488-conjugated) and rabbit Sar1 from Millipore (Billerica, MA); mouse GPR15 from R&D Systems (Minneapolis, MN); mouse calnexin and rabbit ERGIC-53 from Thermo Scientific (Rockford, IL); rabbit BiP/GRP78 from Abcam (Cambridge, MA); mouse GM130 and mouse p230 from BD Biosciences; rabbit β-COP from Affinity BioReagent (Golden, CO); mouse GST from NeuroMab (Davis, CA); Cy3-conjugated goat anti-mouse IgG and anti-rabbit IgG from Invitrogen; and HRP-conjugated goat anti-mouse and goat anti-rabbit IgG from Vector Laboratories (Burlingame, CA).

Cell Culture and Transfection—HEK293 cells were maintained in 50% DMEM/50% Ham’s F-12 medium containing 10% FBS, 2 mm L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were cultured in DMEM with 10% FBS. Transient transfection of plasmids was performed using Mirus TransIT-LT1 (Mirus Bio, Madison, WI) according to the instructions of the manufacturer.

Flow Cytometry (FCM)—Transfected HEK293 cells were collected by gentle flushing and washed with Hank’s balanced salt solution supplemented with 1% BSA (staining buffer). All Ab staining and washing thereafter were performed in the staining buffer on ice. For surface staining of GPR15, cells were incubated for 20 min with GPR15 Ab that was freshly labeled with R-phycocerythrin using a Zenon labeling kit (Invitrogen). After washing, the stained cells were fixed with 1% PFA and analyzed by Cell Lab Quanta SC (Beckman Coulter, Brea, CA). For quantitative comparison of the surface fluorescence intensity between the GPR15 constructs, the median values for the entire cell populations were determined by using FlowJo software (Tree Star, Inc., Ashland, OR).

Subcellular Fractionation—Homogenates of HEK293 cells transfected with HA-GPR15 constructs were fractionated by density gradient centrifugation using OptiPrep (Axis-Shield, Dundee, Scotland) according to the instructions of the manufacturer. Briefly, cells from five 10-cm dishes were resuspended in 1.2 ml of homogenization buffer (containing 10 mN HEPES (pH 7.4), 0.25 M sucrose, and protease inhibitors) and homogenized by 20 strokes in a Dounce homogenizer followed by three passages through a 30-gauge needle. Nuclei and unbroken cells were pelleted by two centrifugations at 1000 × g for 5 min. 800 μl of postnuclear supernatant was loaded onto the top of a discontinuous 6, 8, 10, and 11–28% (with 1% increment) (w/v) Optiprep gradient comprising the layers of 200 μl of volume for each density. After centrifugation at 170,000 × g for 2.5 h with an MLS-50 rotor (Beckman Coulter), a total of 24–26 fractions were collected by dripping from the bottom of the tube at about 160 μl/fraction. The collected proteins were centrifuged in PBS at 100,000 × g for 30 min with a TLA55 rotor (Beckman Coulter), and the pellets were resuspended with 2× sample buffer containing β-mercaptoethanol. The sample fractions of odd numbers were resolved by SDS-PAGE and immunoblotted for HA and the membrane markers.

Immunocytochemistry—HeLa cells transfected with Myc-GPR15 constructs in the chamber slides were fixed with Cytofix/Cytoperm buffer (BD Biosciences) for 15 min at 4 °C and permeabilized with −20 °C chilled 90% methanol for 5 min at room temperature. Cells were first stained with Abs to the intracellular markers overnight at 4 °C, followed by Cy3-labeled secondary Abs for 1 h at 4 °C. Cells were then incubated with AF488-labeled Myc Ab for 1 h at 4 °C and then counterstained with Hoechst 33342 (Invitrogen) for 10 min before mounting. Images were collected with a Zeiss LSM 700 laser scanning confocal microscope using Zen software (Zeiss). The images stained for GPR15 and marker proteins were also analyzed for colocalization by ImageJ using the colocalization plug-in. For
this analysis, the same threshold setting was used for WT and R310/311A mutant images.

**Immunoprecipitation**—The transfected HEK293 cells were washed with PBS once and lysed with lysis buffer (0.5% IGE-PAL, 25 mM Tris, 150 mM NaCl (pH 7.5)) containing protease inhibitors for 20 min at 4 °C. After centrifugation for 20 min at 11,000 × g, the supernatant was mixed with 2–5 μg of appropriate Abs and protein A- or protein G-conjugated agarose beads (Invitrogen). After overnight incubation at 4 °C, the beads were washed four times with lysis buffer, and then the immunoprecipitated proteins were eluted by incubating the beads with 2× sample buffer.

**SDS-PAGE and Western Blot Analyses**—The protein samples resolved by 10 or 12% SDS-PAGE were transferred to nitrocellulose membranes and blocked with skim milk. Membranes were incubated with primary Abs for 1 h at room temperature or overnight at 4 °C and then with corresponding secondary Abs conjugated with HRP. Blot signals were obtained using ECL substrates (Thermo Scientific) and collected by exposing to film or with a Chemi-Doc XRS system (Bio-Rad, Hercules, CA). Band intensity was determined using Quantity One software (Bio-Rad).

**Purification of His-Sar1**—His-tagged Sar1A was purified from *Escherichia coli* using Talon metal affinity resin (Clontech, Mountain View, CA) according to the instructions of the manufacturer. Briefly, *E. coli* strain BL21 transformed with His-Sar1A vector and induced by isopropyl 1-thio-β-D-galactopyranoside was lysed by sonication on ice, and the centrifugation supernatant was incubated with Talon resin. After an extensive wash, the immobilized His-Sar1 was eluted by imidazole and then desalted. The proteins were tested for the purity on SDS-PAGE and used for the binding assay.

**Sar1 Binding Assay**—Lysates from HEK293 cells transfected with vector alone or HA-GPR15 constructs were subjected to immunoprecipitation as described above by using HA Ab and protein A beads. The washed beads were incubated with 2 μg of purified recombinant His-Sar1 in PBS for 60 min, washed, and then incubated with 1 mg/ml of HA peptide (Thermo Fisher) for 15 min to elute the HA-GPR15 proteins. The eluant was analyzed on SDS-PAGE and immunoblotted for Sar1 and HA-GPR15.

**Sec23 and Sec24 Binding Assays**—HEK293 cells were cotransfected with GST-tagged GPR15 tail (amino acids 306–360, WT, or R310/311A) and Myc-tagged Sec23A or GFP-tagged Sec24D. The cell lysates were incubated with glutathione beads (Thermo Scientific) for 2 h at 4 °C. The pulled-down proteins were eluted in sample buffer, resolved by SDS-PAGE, and blotted for GST and Myc or GFP.

**Frequency Analysis of MPBRs**—Protein classification and topology prediction was performed on the basis of the human membrane proteome dataset published in Ref. 19 and the Universal Protein Resource (UniProt). The sequence logo was created using WebLogo version 2.8.2 with “frequency plot” selected.

**RESULTS**

Arg310–Arg311 Are Necessary for Optimal Cell Surface Expression of GPR15—Our previous study demonstrated that the RRX-type dibasic motif (Arg352–Arg354) in the distal C terminus of GPR15 mediates the COPI-mediated retrograde transport of the receptor (15). We were curious whether another pair of C-terminal Arg residues located proximal to the 7th TM segment (Arg310–Arg311, Fig. 1A) was also involved in the vesicular trafficking of GPR15. To investigate the role of MPBRs, we created the mutant GPR15 in which these Arg residues were mutated to Ala (R310/311A). The R310/311A mutant was expressed in HEK293 cells, and the cell surface expression level was examined by FCM. The surface expression of R310/311A mutant was almost abolished (Fig. 1B). We have reported that GPR15 protein undergoes O-linked glycosylation and that the mature glycosylated form is distinguishable by slower migration on SDS-PAGE (15). The mature form is completely lost in the R310/311A mutant (Fig. 1C), which is consistent with the loss of surface expression. To see whether the charge of these residues is relevant, we tested more GPR15 constructs with Arg310–Arg311 substituted by Lys or His. The Arg and Lys residues are positively charged amino groups at physiological pH, but His has a pKₐ of 6 and is only partially charged at neutral pH. GPR15 tolerate the double mutation to Lys (R310/311K) relatively well, with about 50% reduction of surface expression, whereas the double mutation to His (R310/311H) resulted in more than 80% of reduction (Fig. 1, D and E). The total expression levels of these constructs appeared to be similar (Fig. 1F).

In addition, a single Ala mutation on either Arg310 (R310A) or Arg311 (R311A) showed similar effects to that of the double His mutation. These results are consistent with the idea that the net positive charge of the amino acid 310 and 311 positions is critical for the optimal cell surface expression of GPR15.

**Mutation of Arg310–Arg311 Does Not Alter Topology or Assembly of GPR15**—One possible reason for the inefficient surface trafficking of R310/311A mutant could be that the disturbance of MPBRs severely altered the structure of GPR15 and caused incorrect folding and/or incomplete subunit assembly. To examine whether the protein topology was affected by the R310/311A mutation, we took advantage of the fact that the GPR15 C-terminal tail binds to a cytosolic protein 14-3-3 in a phosphorylation-dependent manner (15). If the orientation of the polypeptide chain across the 7th TM segment is reversed, *i.e.* the C terminus in the ER lumen, we would expect that the GPR15 C-terminal tail is not accessible to cytoplasmic Ser/Thr kinases and 14-3-3 proteins. However, the coimmunoprecipitation assay showed strong 14-3-3 association with the R310/311A mutant (Fig. 1B, center panel). This analysis, the same threshold setting was used for WT and R310/311A mutant images.

**RESULTS**

Arg310–Arg311 Are Necessary for Optimal Cell Surface Expression of GPR15—Our previous study demonstrated that the RRX-type dibasic motif (Arg352–Arg354) in the distal C terminus of GPR15 mediates the COPI-mediated retrograde transport of the receptor (15). We were curious whether another pair of C-terminal Arg residues located proximal to the 7th TM segment (Arg310–Arg311, Fig. 1A) was also involved in the vesicular trafficking of GPR15. To investigate the role of MPBRs, we created the mutant GPR15 in which these Arg residues were mutated to Ala (R310/311A). The R310/311A mutant was expressed in HEK293 cells, and the cell surface expression level was examined by FCM. The surface expression of R310/311A mutant was almost abolished (Fig. 1B). We have reported that GPR15 protein undergoes O-linked glycosylation and that the mature glycosylated form is distinguishable by slower migration on SDS-PAGE (15). The mature form is completely lost in the R310/311A mutant (Fig. 1C), which is consistent with the loss of surface expression. To see whether the charge of these residues is relevant, we tested more GPR15 constructs with Arg310–Arg311 substituted by Lys or His. The Arg and Lys residues are positively charged amino groups at physiological pH, but His has a pKₐ of 6 and is only partially charged at neutral pH. GPR15 tolerated the double mutation to Lys (R310/311K) relatively well, with about 50% reduction of surface expression, whereas the double mutation to His (R310/311H) resulted in more than 80% of reduction (Fig. 1, D and E). The total expression levels of these constructs appeared to be similar (Fig. 1F).

In addition, a single Ala mutation on either Arg310 (R310A) or Arg311 (R311A) showed similar effects to that of the double His mutation. These results are consistent with the idea that the net positive charge of the amino acid 310 and 311 positions is critical for the optimal cell surface expression of GPR15.

**Mutation of Arg310–Arg311 Does Not Alter Topology or Assembly of GPR15**—One possible reason for the inefficient surface trafficking of R310/311A mutant could be that the disturbance of MPBRs severely altered the structure of GPR15 and caused incorrect folding and/or incomplete subunit assembly. To examine whether the protein topology was affected by the R310/311A mutation, we took advantage of the fact that the GPR15 C-terminal tail binds to a cytosolic protein 14-3-3 in a phosphorylation-dependent manner (15). If the orientation of the polypeptide chain across the 7th TM segment is reversed, *i.e.* the C terminus in the ER lumen, we would expect that the GPR15 C-terminal tail is not accessible to cytoplasmic Ser/Thr kinases and 14-3-3 proteins. However, the coimmunoprecipitation assay showed strong 14-3-3 association with the R310/311A mutant, which was comparable with that of WT GPR15 (Fig. 2A, center panel). In addition, the presence of a dimeric form of the R310/311A mutant (Fig. 2A, top panel) indicates that this mutation did not significantly affect the receptor assembly. Thus, the inefficient maturation of the R310/311A mutant does not likely arise from its global structural change that could cause abnormal folding and/or assembly.

GPR15 is subjected to Golgi-to-ER retrograde transport by the interaction with COPI proteins via an RRX-type dibasic motif at the distal C terminus (Arg352–Arg354) (15). The RRX motif has been thought to work as a marker for the membrane protein quality control system by being exposed upon misfolding and/or misassembly and recruiting COPI for cargo retrieval to the ER (20, 21). We tested the possibility that the R310/311A mutation somehow made the receptor more susceptible to COPI-dependent ER retrieval. As a positive control, we also created a GPR15, of which the last C-terminal 10 residues were...
replaced with those from the yeast WBP1 protein (KKLETFK-KTN), an ER-resident type I TM protein with an extreme C-terminal ER retrieval motif (KKXX-COOH) that binds to COPI (22, 23). The fusion of the KKXX motif (GPR15-KKXX) abolished cell surface expression of GPR15 (Fig. 2B, top panel), and this was consistent with the lack of the mature form and robust association with β-COP protein (Fig. 2B, fourth lane). In contrast, the R310/311A mutant showed no detectable increase of β-COP association when compared with WT GPR15 (Fig. 2B, second and third lanes). These results suggest that the inefficient maturation of the R310/311A mutant is not due to enhanced COPI-mediated retrograde transport to the ER.

**Figure 1.** Arg310–Arg311 are necessary for optimal cell surface expression of GPR15. A, schematic of the C terminus of human GPR15. B, surface expression of GPR15 WT and the R310/311A mutant. HEK293 cells expressing GPR15 proteins were analyzed for surface expression by FCM. The histograms of transfected cells (filled areas) were overlaid with those of pCDNA3.1 vector-transfected cells (unfilled areas). The x axis indicates the fluorescence intensity in a logarithmic scale, and the y axis indicates the cell number. Values in the histograms indicate the median of the fluorescence intensity determined by FlowJo software for the entire population of analyzed cells. C, protein expression of GPR15. Total lysates from Myc-GPR15-transfected cells were resolved by SDS-PAGE and immunoblotted for Myc. Mature (O-glycosylated) and immature forms of GPR15 are indicated. D, surface expression of GPR15 with different mutations in Arg310, Arg311. Cells transfected with the indicated Myc-GPR15 constructs were analyzed for surface GPR15 signals by FCM. E, quantification of surface GPR15 signals. The median values are shown as mean ± S.D. of triplicate samples from the representative experiment. F, total expression levels of GPR15 proteins. Total lysates from HEK293 cells transfected with the indicated Myc-tagged GPR15 constructs were immunoblotted for Myc (upper panel). The data are representative of three experiments.

**Mutation of Arg310–Arg311 Redistributes GPR15 toward the ER**—To determine the intracellular localization of the R310/311A mutant, we performed a subcellular fractionation assay. The homogenates from HEK293 cells expressing HA-tagged GPR15 were separated by density gradient centrifugation using OptiPrep, and the proteins in the collected fractions were immunoblotted for HA and the marker proteins for ER (calnexin), ERGIC (ERGIC-53), and cis-Golgi (GM130). Fig. 3A shows the representative data from four different experiments. The results of the other experiments are shown in supplemental Fig. 1). The relative signal of HA in each fraction was plotted to compare the overall distribution of WT and the mutant GPR15.
FIGURE 2. Mutation of Arg310–Arg311 does not alter topology or subunit assembly of GPR15. A, GPR15 association with 14-3-3 proteins. Myc-GPR15 proteins were expressed in HEK293 cells and immunoprecipitated (IP) with Myc Ab. The eluants resolved by the SDS-PAGE were immunoblotted for Myc (top panel) and 14-3-3 (center panel). The bottom panel shows the input GPR15 signal in the total lysate. m, mature form; imm, immature form. B, surface expression and COPI interaction of GPR15 mutants. The histograms (top panel) of transfected cells (filled areas) were overlaid with those of vector-transfected cells (unfilled areas). Myc-GPR15 proteins were immunoprecipitated with Myc Ab, and the eluants resolved by SDS-PAGE were immunoblotted for β-COP, Myc, and 14-3-3 (lower panels). Data are representative of three experiments.

FIGURE 3. Mutation of Arg310–Arg311 redistributes GPR15 protein toward the ER-enriched membrane fractions. A, the homogenates from HEK293 cells expressing HA-tagged GPR15 WT or R310/311A were separated by discontinuous density gradient centrifugation using Optiprep (6–28%). After centrifugation at 170,000 × g for 2.5 h, the gradient was collected by dripping from the bottom of the tube. The pellets obtained by further centrifugation at 100,000 × g for 30 min were resuspended with sample buffer. The sample fractions of odd numbers were subjected to SDS-PAGE followed by immunoblotting of HA and markers for ER (Calnexin), ERGIC (ERGIC-53), and cis-Golgi (GM130). CL, control lysate of non-transfected HEK293 cells. B, the HA-GPR15 signal was quantified by molecular imager, and the relative signal intensity from each fraction was plotted. Representative data from four different experiments are shown. See the results of the other three experiments in supplemental Fig. 1). C, the relative percentages of HA-GPR15 signals in the calnexin-positive fractions were compared between the WT and the R310/311A mutant. The values are mean ± S.D. from four experiments. The significance of differences was analyzed by Student’s t test.
Membrane-proximal Basic Residues in GPCR Trafficking

5, A and B). In addition, the triple Ala mutant was still readily colocalized with the ER marker BiP, whereas the R352A single mutant was mostly out of the ER (Fig. 5C). Together, these results indicate that the mutation of Arg310-Arg311 significantly reduces the rate of ER export of GPR15.

Arg310-Arg311 Are Not Necessary for the Interaction of the GPR15 C-terminal Tail with ER Export Machineries—Many of the peptide sequences that have been reported as “ER export” signals are capable of interacting with ER export machineries such as COPII coat proteins and the GTPase that recruits coat proteins (9, 10). The MPBRs in the Golgi-resident glycosyltransferases and ADRA2B have been shown to be necessary for binding to Sar1 GTPase and Sec24D protein, respectively (13, 14). We were curious whether the Arg310-Arg311 residues of GPR15 also play such a role. First, we addressed whether GPR15 is a cargo of the COPII-dependent anterograde transport by testing the effect of coexpression of a dominant negative Sar1 GTPase, H79G. Sar1 H79G is a GTP-locked, constitutively active form of Sar1, and its overexpression is known to block ER-to-Golgi transport of cargo proteins in the secretory pathway (24). Coexpression of Sar1 H79G markedly reduced cell surface expression and O-glycosylation (Fig. 6, A and B) of GPR15, whereas the WT Sar1 did not. These results suggest that GPR15 is exported from the ER in a canonical COPII-dependent manner. There is a possibility that expression of dominant negative Sar1 inhibited the ER-to-Golgi transport of the O-glycosylation enzymes and thereby reduced the O-glycosylation of GPR15 in the Golgi. If so, and if O-glycosylation per se is required for cell surface trafficking of GPR15, the expression of Sar1 H79G could be also indirectly inhibiting the surface trafficking of GPR15. However, this is likely not the case because the Ala mutant in the N-terminal O-glycosylation sites (Ser and Thr at amino acids 6, 7, 16, 17, and 20) that completely lacks the mature form of GPR15 (15) still largely maintains its cell surface expression (data not shown). Thus, possible impairment of the transport of the O-glycosylation machinery to the Golgi by Sar1 H79G expression will not affect our interpretation that the ER export of GPR15 is COPII-dependent.

On this basis, we next examined whether Arg310-Arg311 are involved in the binding of COPII component proteins to GPR15. The HA-tagged WT and R310/311A mutant GPR15 proteins were immunoprecipitated and then incubated with recombinant His-tagged Sar1 purified from bacteria. GPR15 proteins were eluted from the protein A beads by competitive HA peptide, and the eluants were probed for Sar1 and GPR15. Fig. 6C shows the representative data showing similar binding of Sar1 to the WT and the R310/311A mutant. There was no Sar1 signal for the control sample (Fig. 6C, second lane) where cells transfected with the vector alone were processed in parallel, indicating the specificity of the Sar1 signal to GPR15. Quantitative analysis of the band intensities from three experiments showed no statistically significant difference in Sar1 binding to the WT and the mutant GPR15 (Fig. 6D). We next tested the binding of COPII coat proteins to the GST fusions coexpressed in the cell. The entire C-terminal cytoplasmic tail encoding amino acids 306–360 of GPR15 was fused to GST, coexpressed

![Figure 4](image-url.com)
Membrane-proximal Basic Residues in GPCR Trafficking

We were curious how frequently MPBRs like Arg^310^-Arg^311^ are present in human membrane proteins, particularly in the GPCR family. We aligned the membrane-proximal ten amino acid residues in the C-terminal tail from human GPCRs listed in the published membrane protein database (19) and examined the frequency of amino acids in each position relative to the TM segment (Fig. 7, see all sequences in the supplemental Table). Because the olfactory receptors (including putative ones) represent a majority of the GPCR family, the results were shown separately for non-olfactory (Fig. 7A, top panel) and olfactory GPCRs (center panel). The analysis of 365 non-olfactory GPCRs revealed that Arg and Lys are most frequent in the positions that are 5, 6, 9, and 10 amino acids away from the seventh TM segment. This is the same for GPR15, in which Arg^310^-Arg^311^ are positioned in the fifth and sixth positions (Fig. 7B). The olfactory receptors show a more conserved pattern in the distribution of Arg and Lys at particular positions such as 1, 3, 6, and 10 amino acids away from the TM. The single-TM proteins that are categorized as “receptors” (n = 296, excluding type II TM proteins) show a much broader preference of basic residues in the membrane proximity; i.e. either Arg or Lys was most frequent in all positions within the 10 residues from the TM segment (Fig. 7A, bottom panel). This might be due to the larger diversity of protein structures in this group as compared with the GPCR family.

Having the relatively conserved C-terminal MPBRs in GPCRs, we set out to test whether these MPBRs are also required for cell surface trafficking in GPCRs other than GPR15. We chose more than ten proteins from the class A GPCR family and selected those whose surface expression was detectable by the N-terminally tagged Myc epitope using FCM. Fig. 7C shows the results with eight different GPCRs for which the effect of the Ala mutation in the C-terminal MPBRs (B) on cell surface expression were tested. The majority showed a marked reduction of cell surface expression by the Ala mutation. Notably, the effects of the mutation on surface expression level was correlated with the numbers of the MPBRs mutated in ADRA2B (compare double and quadruple Ala mutants). Interestingly, some GPCRs, such as GPR39 and NPFRR1, showed only a modest reduction of surface expression by the MPBRs mutation. This suggests that the role of C-terminal MPBRs in GPCRs trafficking may vary significantly, depending on the proteins.

**DISCUSSION**

The ER-to-Golgi transport in most secretory proteins is coordinated by COPII and COPI coats that direct anterograde and retrograde movement of vesicles, respectively. Selective packaging of cargos into these vesicles can be facilitated by the presence of distinct sequence signals on cargos that are recognized by the coat proteins (10, 25, 26). Our previous study on GPR15 revealed that an RRX-type dibasic signal at the distal C terminus (Arg^352^-Arg^354^) negatively regulated the cell surface transport of GPR15 by the interaction with COPI proteins (15). In this work we addressed the question whether the Arg^310^-Arg^311^- dibasic signal at the membrane-proximal C terminus would also play roles in cell surface trafficking. We found that the specific mutation of these MPBRs to Ala abolished cell surface expression and O-glycosylation of GPR15 (Fig. 1). The net positive charge of the residues at amino acids 310 and 311 was critical for their role in cell surface expression. This indicates that the cytoplasmic MPBRs, which are known to dictate the orientation of newly synthesized polypeptides during ER insertion (positive-inside rule) (16, 18), can serve an additional crucial role in vesicular trafficking. One potential caveat with our approach of using the Ala mutants may be that the mutation of MPBRs could cause a global change in the protein structure, such as topology and subunit assembly, and therefore would make it difficult to dissect the role of MPBRs in vesicular trafficking from that in the receptor biogenesis in the ER. Although we cannot completely exclude this possibility, our biochemical

with Myc-Sec23A, and pulled down with glutathione beads. The GST fusion with the R310/311A mutation was associated with more Sec23A than the WT fusion (Fig. 6E). We also tested the binding of these GST fusions to GFP-fused Sec24D, which was used recently to show that the Sec24 interaction with ADRA2B requires the MPBRs in the third cytoplasmic loop (14). Similar to the Sec23A results, GFP-Sec24D showed stronger binding to the R310/311A mutant fusion protein than to the WT fusion (Fig. 6F). Collectively, these results indicate that Arg^310^-Arg^311^- do not play a positive role in the interaction of GPR15 with COPII components and, hence, suggest that the inefficient exit of the R310/311A mutant may not be due to the simple loss of interaction with COPII machinery proteins.
characterization indicated that the R310/311A mutant maintains a topology and subunit assembly status identical to those of WT GPR15 (Fig. 2). Moreover, the fact that some of the other GPCRs tested in Fig. 7 showed a modest effect of MPBR mutation on surface expression also implies that the mutation of C-terminal MPBRs will not automatically cause folding defects in GPCRs.

Our finding that Arg^{310}-Arg^{311} of GPR15 are necessary for surface trafficking is essentially consistent with the role of MPBRs reported previously in several other proteins. These include CCR5 and MCH1R, whose surface expression was reduced by deletion or mutation of the C-terminal MPBRs (11, 12). In these studies, the precise mechanism underlying the defects in surface trafficking of the receptors was not shown.
However, the studies by Giraudo et al. (13) demonstrated that the N-terminal MPBRs in the Golgi-resident glycosyltransferases were necessary for the interaction with Sar1 GTPase and that mutation of these MPBRs redistributed the enzymes to the ER. In addition, a more recent study showed that the MPBRs in the third intracellular loop of the ADRA2B were necessary for Sec24D binding and the optimal ER export of the receptor (14). In contrast to those studies, our results suggest that the mutation of C-terminal MPBRs results in substantially delayed exit of GPR15 from the ER. What are the possible mechanisms? Our binding assays using full-length GPR15 and GST fusions encoding the C-terminal tail of GPR15 did not show evidence that Arg310-Arg311 play a positive role in the interaction with COPII components (Fig. 6). It is possible, however, that the MPBRs of GPR15 may contribute optimally to the interaction with COPII proteins only by cooperating with other remotely positioned sequence signals in the native conformation. Indeed, this notion is true for the post-Golgi trafficking signal of the Kir2.1 channel, in which the two independent sequence signals located in the N-terminal and C-terminal tails are recognized as a single signal patch by the AP1 adaptor protein only when the channel is folded in a tertiary structure (27).
Membrane-proximal Basic Residues in GPCR Trafficking

Another interesting notion comes from studies on CCR5. The C-terminal MPBRs in CCR5 were shown to support anterograde trafficking in cooperation with the downstream Cys residues (11). The C-terminal Cys residues that are conserved in the majority of GPCRs are the sites of palmitoylation, which is thought to mediate the interaction of the C-terminal tail with membranes to form an additional cytoplasmic loop (28). The reduced surface expression in various GPCRs with a C-terminal Cys substitution (29–32) indicates that Cys palmitoylation is a vital process for correct folding and trafficking. On the basis of this assumption, the C-terminal MPBRs may contribute critically to this process by possibly making initial contact with membranes through electrostatic interaction with the negatively charged head group of the membrane phospholipids (11). However, this model may not apply universally to all GPCRs because receptors such as ADRA2C, which do not have Cys in the C-terminal tail, also showed reduced surface expression by MPBR mutation (Fig. 7). The precise mechanistic role of C-terminal MPBRs in the ER export of GPR15 awaits further investigation. Because the sorting signal that is necessary and sufficient for COPII binding is not always sufficient for actually mediating ER export (33), more functional assays may be necessary to determine whether these MPBRs are involved in the interaction with COPII components.

The notion that C-terminal MPBRs are necessary for the anterograde trafficking of membrane proteins raises an intriguing question. Why would MPBRs that often resemble the COPII-binding dibasic motif, like RXR, not serve as an ER retrieval motif? Our previous study revealed the presence of differential functional zoning for the RXR signal and C-terminal KXXX signal (34). The ER retrieval activity of the RXR signal was decreased when it was positioned closer to the TM segment, whereas the opposite effect was observed for the KXXX signal. The loss of RXR signal activity in membrane proximity has also been supported by other studies using different membrane proteins (35, 36). This may be related to the differential binding property of the RXR and KXXX motifs to the COPII proteins, as has been revealed by structural studies (37). On this basis, we speculate that the basic residues proximal to the TM segment will not function as an ER retrieval signal, possibly because of their inefficient access to the COPII proteins. This may allow MPBRs to function as a determinant of protein topology (positive-inside rule) and/or an anterograde trafficking signal, which should be distinguished from other clusters of basic residues in more distal positions.

The analysis of C-terminal MPBRs revealed the prevalence of basic residues in the membrane-proximal region of human GPCRs as well as other receptor proteins (Fig. 7), which is consistent with the positive-inside rule. However, our studies on the basis of the R310/311A mutation shows that these MPBRs are not required critically for determining the topology of GPR15. This is consistent with other reports studying the role of MPBRs (11–14). Thus, the positive-inside rule is not always absolute.

Our studies on different GPCRs showed marked effects of MPBR mutation on their surface expression (Fig. 7). Although a more detailed characterization of individual receptors such as folding status and intracellular localization will be necessary to fully interpret these results, it is conceivable that the C-terminal MPBRs play a critical role in anterograde trafficking in a broad range of GPCRs and, presumably, in other TM proteins. However, the fact that some GPCRs showed only a modest effect (i.e. GPR39 and NPFFR1) of MPBR mutation also suggests that the functional role of MPBRs varies significantly, depending on the structural attributes of the individual receptors, such as the flanking sequences and the presence of other anterograde trafficking signals. Further investigation on membrane proteins of different structures and functions will provide new mechanistic insights into the role of MPBRs in membrane protein trafficking.

Acknowledgments—We thank Drs. Jack Kaplan and Edward Maryon (University of Illinois at Chicago, Chicago, IL) for generous support and advice throughout this study.

REFERENCES
1. Dunham, J. H., and Hall, R. A. (2009) Enhancement of the surface expression of G protein-coupled receptors. Trends Biotechnol. 27, 541–545
2. Drake, M. T., Shenoy, S. K., and Lefkowitz, R. J. (2006) Trafficking of G protein-coupled receptors. Circ. Res. 99, 570–582
3. Wolfe, B. L., and Trejo, J. (2007) Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. Traffic 8, 462–470
4. Gurevich, V. V., and Gurevich, E. V. (2008) GPCR monomers and oligomers. It takes all kinds. Trends Neurosci. 31, 74–81
5. Hanyaloglu, A. C., and von Zastrow, M. (2008) Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu. Rev. Pharmacol. Toxicol. 48, 537–568
6. Barlowe, C. (1998) COPII and selective export from the endoplasmic reticulum. Biochim. Biophys. Acta 1404, 67–76
7. Glick, B. S. (2001) ER export. More than one way out. Curr. Biol. 11, R361–363
8. Palmer, K. J., and Stephens, D. J. (2004) Biogenesis of ER-to-Golgi transport carriers. Complex roles of COPII in ER export. Trends Cell Biol. 14, 57–61
9. Zanetti, G., Pahuja, K. B., Studer, S., Shim, S., and Scheckman, R. (2012) COPII and the regulation of protein sorting in mammals. Nat. Cell Biol. 14, 20–28
10. Barlowe, C. (2003) Signals for COPII-dependent export from the ER. What’s the ticket out? Trends Cell Biol. 13, 295–300
11. Venkatesan, S., Petrovic, A., Locati, M., Kim, Y. O., Weissman, D., and Murphy, P. M. (2001) A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression. J. Biol. Chem. 276, 40133–40145
12. Tetsuka, M., Saito, Y., Imai, K., Doi, H., and Maruyama, K. (2004) A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression. J. Biol. Chem. 276, 40133–40145
13. Giraud, C. G., and Maccioni, H. J. (2003) Endoplasmic reticulum export of glycosyltransferases depends on interaction of a cytoplasmic dibasic motif with Sar1. Mol. Biol. Cell 14, 3753–3766
14. Dong, C., Nichols, C. D., Guo, J., Huang, W., Lambert, N. A., and Wu, G. (2012) A triple Arg motif mediates α(2B)-adrenergic receptor interaction with Sec24C/D and export. Traffic 13, 857–868
15. Okamoto, Y., and Shikano, S. (2011) Phosphorylation-dependent C-terminal binding of 14-3-3 proteins promotes cell surface expression of HIV co-receptor GPR15. J. Biol. Chem. 286, 7171–7181
16. Higy, M., Junne, T., and Spiess, M. (2004) Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry 43, 12716–12722
17. Dowhan, W., and Bogdanov, M. (2009) Lipid-dependent membrane protein topogenesis. Annu. Rev. Biochem. 78, 515–540
18. Nilsson, J., Persson, B., and von Heijne, G. (2005) Comparative analysis of amino acid distributions in integral membrane proteins from 107 ge-
nomes. Proteins 60, 606–616

19. Almén, M. S., Nordström, K. J., Fredriksson, R., and Schiöth, H. B. (2009) Mapping the human membrane proteome. A majority of the human membrane proteins can be classified according to function and evolutionary origin. BMC Biol. 7, 50

20. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. Neuron 22, 537–548

21. Yuan, H., Michelsen, K., and Schwappach, B. (2003) 14-3-3 dimers probe the assembly status of multimeric membrane proteins. Curr. Biol. 13, 638–646

22. Cosson, P., and Letourneur, F. (1994) Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science 263, 1629–1631

23. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 9, 3153–3162

24. Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C., and Balch, W. E. (1998) Cargo selection by the COPII budding machinery during export from the ER. J. Cell Biol. 141, 61–70

25. Wendeler, M. W., Paccaud, J. P., and Hauri, H. P. (2007) Role of Sec24 isoforms in selective export of membrane proteins from the endoplasmic reticulum. EMBO Rep. 8, 258–264

26. Sato, K. (2010) Selective protein export from the ER mediated by COPII-coated vesicles. Seikagaku 82, 1011–1020

27. Ma, D., Taneja, T. K., Hagen, B. M., Kim, B. Y., Ortega, B., Lederer, W. J., and Welling, P. A. (2011) Golgi export of the Kir2.1 channel is driven by a trafficking signal located within its tertiary structure. Cell 145, 1102–1115

28. Chini, B., and Parenti, M. (2009) G-protein-coupled receptors, cholesterol and palmitoylation. Facts about fats. J. Mol. Endocrinol. 42, 371–379

29. Fukushima, Y., Saitoh, T., Anai, M., Ogihara, T., Iinukai, K., Funaki, M., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., Ishikawa, T., Takata, K., Nagai, R., Omata, M., and Asano, T. (2001) Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. Biochim. Biophys. Acta 1539, 181–191

30. Karnik, S. S., Ridge, K. D., Bhattacharya, S., and Khorana, H. G. (1993) Palmitoylation of bovine opsin and its cysteine mutants in COS cells. Proc. Natl. Acad. Sci. U.S.A. 90, 40–44

31. Zhu, H., Wang, H., and Ascoli, M. (1995) The lutropin/choriogonadotropin receptor is palmitoylated at intracellular cysteine residues. Mol. Endocrinol. 9, 141–150

32. Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachererie, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. J. Biol. Chem. 276, 31936–31944

33. Otté, S., and Barlowe, C. (2002) The Erv41p-Erv46p complex. Multiple export signals are required in trans for COPII-dependent transport from the ER. EMBO J. 21, 6095–6104

34. Shikano, S., and Li, M. (2003) Membrane receptor trafficking. Evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localization signals. Proc. Natl. Acad. Sci. U.S.A. 100, 5783–5788

35. Khalil, H., Brunet, A., and Thibodeau, J. (2005) A three-amino-acid-long HLA-DRβ cytoplasmic tail is sufficient to overcome ER retention of invariant-chain p35. J. Cell Sci. 118, 4679–4687

36. Gassmann, M., Haller, C., Stoll, Y., Abdel Aziz, S., Biermann, B., Mosbacher, J., Kaupmann, K., and Bettler, B. (2005) The RXR-type endoplasmic reticulum-retention/retrieval signal of GABAB1 requires distant spacing from the membrane to function. Mol. Pharmacol. 68, 137–144

37. Michelsen, K., Schmid, V., Metz, J., Heusser, K., Liebel, U., Schwede, T., Spang, A., and Schwappach, B. (2007) Novel cargo-binding site in the β and Δ subunits of coatomer. J. Cell Biol. 179, 209–217