Phosphorylation-dependent C-terminal Binding of 14-3-3 Proteins Promotes Cell Surface Expression of HIV Co-receptor GPR15*

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Membrane trafficking is dictated by dynamic molecular interactions involving discrete determinants in the cargo proteins and the intracellular transport machineries. We have previously reported that cell surface expression of GPR15, a G protein-coupled receptor (GPCR) that serves as a co-receptor for HIV, is correlated with the mode III binding of 14-3-3 proteins to the receptor C terminus. Here we provide a mechanistic basis for the role of 14-3-3 in promoting the cell surface expression of GPR15. The Ala mutation of penultimate phospho-Ser (S359A) that abolishes 14-3-3 binding resulted in substantially reduced O-glycosylation and the cell surface expression of GPR15. The surface membrane protein CD8 fused with the C-terminal tail of GPR15S359A mutant was re-localized in the endoplasmic reticulum (ER). In the context of S359A mutation, the additional mutations in the upstream stretch of basic residues (RXR motif) restored O-glycosylation and the cell surface expression. The RXR motif was responsible for the interaction with coatomer protein I (COPI), which was inversely correlated with the 14-3-3 binding and cell surface expression. These results suggest that 14-3-3 binding promotes cell surface expression of GPR15 by releasing the receptor from ER retrieval/retention pathway that is mediated by the interaction of RXR motif and COPI. Moreover, 14-3-3 binding substantially increased the stability of GPR15 protein. Thus 14-3-3 proteins play multiple roles in biogenesis and trafficking of an HIV co-receptor GPR15 to control its cell surface density in response to the phosphorylation signal.

G protein-coupled receptors (GPCRs) represent the largest class of cell surface receptors and play an integral role in an enormous array of biological pathways. The magnitude of GPCR-mediated signaling is dictated by the cell surface density of the receptors. Naturally, genetic mutations that result in deficient cell surface trafficking of GPCRs are correlated with pathophysiology of various human diseases (1). For instance, the majority of mutations in vasopressin 2 receptor (V2R) that result in nephrogenic diabetes insipidus cause ER retention of the receptors (2). On the other hand, the genetic deletion in HIV-1 co-receptor CCR5 (CCR5-Δ32) reduces its cell surface expression and this confers the resistance to viral infection (3). Thus elucidation of molecular mechanisms that regulate cell surface transport of GPCRs will provide important basis for therapeutic intervention as well as for understanding of GPCR biology. However, compared with the extensive studies on the ligand-induced endocytic pathways (4–7), the mechanisms underlying the forward trafficking of GPCRs from the ER to the cell surface are relatively less well understood.

Our previous screening of a random peptide library identified a group of C-terminal sequences that directed the efficient cell surface expression of a reporter membrane protein by specific binding to 14-3-3 proteins (8). The 14-3-3 proteins are a highly conserved family of proteins that usually bind to the phosphopeptide motifs in the target proteins and play central regulatory roles in a wide variety of biological pathways (9–11). One of the emerging roles of 14-3-3 proteins in recent years is the promotion of cell surface transport of membrane proteins (12, 13). The underlying mechanism by which 14-3-3 binding mediates forward trafficking of membrane proteins seems to be diverse and involve multiple different protein interactions (8, 14–16). We have reported that C-terminal (mode III) 14-3-3 binding to GPR15, a putative chemokine receptor and known as a co-receptor for HIV and simian immunodeficiency virus (SIV) (17, 18), is correlated with the efficient surface expression of the receptor (8). However, mechanistic basis for the role of 14-3-3 has not been understood. Here we demonstrate that the phosphorylation-dependent 14-3-3 binding promotes cell surface expression of GPR15 by suppressing the dibasic (RXR) motif-mediated ER localization activity and also by increasing the stability of GPR15. Thus we for the first time demonstrate a critical role of 14-3-3 proteins in controlling the cell surface density of a GPCR.

EXPERIMENTAL PROCEDURES

Plasmids—The human GPR15 gene was cloned in pCMV vectors with N-terminal HA or Myc epitope as described previously (19). For untagged GPR15 constructs, the GPR15 gene was cloned in pCDNA3.1 (+) (Invitrogen, Carlsbad, CA). Human CD8 was either tagged with Myc (inserted after the N-terminal signal sequence) or with HA (inserted after the cytoplasmic tail) and cloned in pCDNA3.1 (+). Site-directed mutagenesis was performed by overlap extension PCR.

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2 The abbreviations used are: GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; FCM, flow cytometry; SIV, simian immunodeficiency virus; COPI, coatomer protein I; IP, immunoprecipitation; Ab, antibody.
**Antibodies (Abs)**—Mouse anti-HA, rabbit anti-HA, rabbit anti-myc, rabbit anti-14-3-3β (reacts to all seven 14-3-3 isoforms), and rabbit anti-β-tubulin were all from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse anti-Myc with or without Alexa Fluor (AF) 488 label was from Millipore (Billerica, MA), mouse anti-human GPR15 was from R&D Systems (Minneapolis, MN), rabbit anti-GPR15 was from Sigma-Aldrich (St. Louis, MO), mouse anti-calnexin, mouse anti-GM130, and mouse anti-p230 were from BD Biosciences (San Jose, CA), rabbit anti-phospho Akt substrate (RXX(S/T)) was from Cell Signaling Technology (Danvers, MA), rabbit Abs against α-COP, β-COP, and γ-COP were from Affinity BioReagent (Golden, CO).

**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. CHO IdID cells were generously provided by Dr. M. Krieger and cultured in Ham’s F-12 with 5% FBS. 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 manufacturer’s instructions.

**Flow Cytometry (FCM)**—Transfected HEK293 cells were collected by gentle flushing and washed with Hanks’ Balanced Salt Solution (HBSS) supplemented with 1% BSA (staining buffer). All the Ab staining and washing thereafter were performed in the staining buffer at 4 °C. For surface staining of GPR15, cells were incubated for 30 min at 4 °C with GPR15 mAb pre-labeled with R-phycocerythrin (PE) using Zenon labeling kit (Invitrogen). For Myc-tagged CD8, myc- AF488 mAb was used for stain. After wash, the stained cells were fixed with 1% PFA and analyzed by Cell Lab Quanta SC (Becton Coulter, Brea, CA). The geometric mean of the cell surface fluorescence from the live cells was obtained by Flowjo software (Tree Star Inc., Ashland, OR).

**Immunocytochemistry**—HeLa cells transfected with Myc-GPR15 or Myc-CD8 fusion constructs in the chamber slides were fixed with Cytofix/Cytoperm buffer (BD Pharmingen) 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 for the intracellular markers overnight at 4 °C, followed by Cy3-labeled secondary Abs (Invitrogen) for 1 h at 4 °C. Cells were then incubated with AF488-Myc mAb for 1 h at 4 °C, and then with Hoechst 33342 (Invitrogen) for 10 min before mounting. For cell surface staining of GPR15 or CD8 in the live cells, the transfected cells were incubated with AF488-Myc mAb at 4 °C for 30 min followed by wash and Hoechst stain. Images were collected with a Zeiss LSM 5 Pascal laser scanning microscope using LSM Image Browser (Zeiss) software.

**Immunoprecipitation (IP)**—For IP of GPR15 from total cell lysate, the transfected cells were washed with PBS once and lysed with lysis buffer (0.5% Igepal, 25 mM Tris, 150 mM NaCl, pH 7.5) containing protease inhibitor cocktails (Pierce) for 20 min at 4 °C. After centrifugation for 20 min at 11,000 × g, the supernatant was mixed with 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 precipitated proteins were eluted by incubating the beads with 2× SDS sample buffer containing β-mercaptoethanol for 30 min at room temperature. For IP of cell surface GPR15, the cells transfected with Myc-GPR15 constructs were first collected and live stained with Myc mAb for 30 min on ice. After washing, the cells were lysed and the centrifuge supernatants were incubated with protein G-beads for 4 h at 4 °C. The beads were washed and the immunoprecipitated proteins were eluted as described for the total cell IP.

**PAGE and Western Blots**—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 overnight at 4 °C and then with corresponding secondary Abs conjugated with horseradish peroxidase (HRP) (Vector Laboratory, Burlingame, CA). Blot signals were obtained using ECL substrates (Pierce) and collected by exposing to film or with a Chemiluminescence System (Bio-Rad). Band intensity was determined using Quantity One® Software (Bio-Rad).

**In Vitro Peptide Pull-down**—Peptides encoding the C-terminal sequence of GPR15 (aa 347–360) with or without phosphorylation at Ser159 were synthesized by Genscript (Piscataway, NJ) and Neo Biosciences (Cambridge, MA). The peptides were covalently coupled to SulfoLink Coupling Gel (Pierce) and incubated with HEK293 cell lysate for 2 h at 4 °C. The pulled-down proteins were eluted with sample buffer, resolved by SDS-PAGE and blotted for 14-3-3 or COP proteins.

**Pulse-chase Assay**—HEK293 cells were transfected with HA–GPR15 constructs in 60 mm dishes. After 48 h, the cells were starved in Met/Cys-free pulse medium for 30 min. Fresh [35S]Met/Cys (Trans [35S], MP Biomedical, Solon, OH) was added to the culture at 100 μCi/ml and the cells were incubated for 10 min at 37 °C, and then washed twice with HBSS at RT. The cells were incubated in the complete medium supplemented with 2 mM of Met and Cys for 1, 3, and 5 h at 37 °C. Cell lysates made at each time point were immunoprecipitated with HA mAb at 4 °C. The eluates were resolved by SDS-PAGE and the dried gels were analyzed for the radioactivity by phosphor imager (Typhoon, GE Helencare, Piscataway, NJ). The GPR15 signals were quantified by ImageQuant software (GE Helathcare).

**Glycosidase Treatment**—All the reagents including enzymes and buffers for this assay were purchased from New England Biolabs (Ipswich, MA). Wt GPR15 proteins were immunoprecipitated with HA mAb and the beads were incubated in G7 buffer containing neuraminidase, endo-α-N-acetylgalactosaminidase (O-glycosidase), β1,4-galactosidase, and β-N-acetylglucosaminidase for 4 days at 37 °C. The eluted proteins were resolved by SDS-PAGE and blotted for GPR15.

**RESULTS**

**Phosphorylation-dependent 14-3-3 Binding Is Necessary and Sufficient for Efficient Surface Expression of GPR15**—Previously we showed that mutation of penultimate Ser159 to Ala (Fig. 1A) abolished 14-3-3 binding and substantially reduced cell surface expression of GPR15 (8). The in vitro pull-down
assay using synthetic peptides encoding GPR15 C terminus clearly shows that 14-3-3 binding is dependent on the phosphorylation of Ser$^{359}$ (Fig. 1B). To test the functional correlation between 14-3-3 binding and receptor surface expression, we designed GPR15 that lacks Ser$^{359}$ but is fused with an R18 peptide sequence (Fig. 1C, GPR15$_{3588}$-R18). The R18 is an unphosphorylated peptide that was isolated as a high affinity 14-3-3 binding sequence by a phage library screen (20), and has no sequence homology to GPR15 C terminus. Its binding to 14-3-3 requires the core WLDLE sequence (20). The co-IP and FCM assays showed that C-terminally fused-R18 sequence mediated the 14-3-3 binding to the receptor (Fig. 1D) and confered the comparable surface expression to that of Wt GPR15 (Fig. 1E). In contrast, fusion of R18 sequence carrying Ala mutations in the core region (Fig. 1C, GPR15$_{3588}$-R18$_{AAA}$) resulted in no 14-3-3 binding and low expression similar to that of GPR15$_{S359A}$ (Fig. 1, D and E). These results suggest that C-terminal 14-3-3 binding is sufficient for conferring the efficient surface expression of GPR15.

Phosphorylation signal plays a key role in a wide variety of protein trafficking pathways. In the N-methyl-D-aspartate (NMDA) receptor NR1, phosphorylation of Ser residues adjacent to the Arg stretch (RRRS$_{9896}^{987}$) by PKA and PKC is thought to allow the receptor for efficient trafficking to synapses (21). Because our S359A mutation abolishes both phosphorylation of Ser$^{359}$ and 14-3-3 binding, it is still possible that in the Wt GPR15, phosphorylation of Ser$^{359}$ itself is sufficient to mediate surface transport of GPR15 regardless of subsequent 14-3-3 binding. To address this possibility, we created GPR15 with three Ala residues extended (Fig. 1C). This AAA extension should perturb the C-terminal mode III 14-3-3 binding motif by shifting the phosphorylatable Ser from −2 to −5 position (22) but will still preserve the Ser/Thr kinase recognition motif. By this we attempted to dissect the effect of phosphorylation alone from that of subsequent 14-3-3 binding. The eluates from IP of GPR15 were blotted with Abs against HA (Fig. 1F, top), phosphorylated RXX(S/T) motif (middle), and the associating 14-3-3 (bottom). As expected, GPR15-AAA showed similar level of phosphorylation signals to that of Wt, but completely lacked 14-3-3 binding. The absence of phospho-RXXS signal in GPR15$_{S351}$ mutant lacking the C-terminal 9 residues confirmed the specificity of this phosphorylation signal (data not shown). Under this condition we observed low surface expression of GPR15-AAA similar to that of GPR15$_{S359A}$ (Fig. 1G). Collectively, our data demonstrate that Ser$^{359}$-dependent 14-3-3 binding is both necessary and sufficient for mediating the efficient surface expression of GPR15.

14-3-3 Binding Promotes ER-to-Golgi Transport of GPR15—GPCRs are well known to undergo various post-translational modifications along the secretory pathway. In SDS-PAGE under the reducing condition, N-terminally HA-tagged GPR15 protein migrated with a molecular mass of ~40–45 kDa, 80–90 kDa, and larger (>150 kDa) that seem to represent monomers, dimers, and oligomers and/or aggregates, respectively (Fig. 2A). The relative intensity of a slower
FIGURE 2. **14-3-3 binding promotes ER-to-Golgi transport of GPR15.** A, immunoblot of Wt and S359A mutant. HA-GPR15 was immunoprecipitated and blotted for GPR15. Asterisks indicate faster (*) and slower (**) migrating bands. B, digestion of GPR15 with O-glycosidase. HA-GPR15 proteins were immunoprecipitated and digested or not with O-glycosidase. The eluted proteins were immunoblotted for GPR15. C, O-glycosylation of GPR15 in ldlD CHO cell. The ldlD cells that had been cultured in the medium supplemented with dialyzed FBS was transfected with Myc-GPR15 constructs and then added or not with GalNAc and galactose. The next day, the total cell lysates were immunoblotted for GPR15. D, O-glycosylation in GPR15 with Ala mutations on N-terminal Ser and Thr. HA-GPR15 with Ala mutations on N-terminal Ser and Thr was expressed, and the immunoprecipitants were blotted for GPR15 and 14-3-3. E, association of 14-3-3 with cell surface GPR15. Total cellular GPR15 and the cell surface pool of GPR15 proteins were separately immunoprecipitated. For cell surface IP, transfected cells were live-stained with Myc mAb, washed, and then lysed. The lysate was incubated with protein G beads, and the eluate was blotted for GPR15 and 14-3-3. **m,** mature; **imm,** immature. F, cell surface expression of GPR15. HeLa cells transfected with Myc-GPR15 were live-stained for surface GPR15 with AF488-Myc mAb (green) and counterstained with Hoechst 33342 (blue). G, colocalization of GPR15 and intracellular markers (calnexin for ER, GM130 for cis-Golgi, and p230 for trans-Golgi). Transfected cells were fixed, permeabilized, and stained for GPR15 and markers followed by counterstain. Representative confocal images of GPR15 (green) and markers (red), and colocalization of these proteins (yellow) are shown. Nuclei are shown in blue. The enlarged images of selected areas (squares) were also shown.
migrating band (**) to the faster migrating band (*) was low in S359A mutant when compared with Wt GPR15 (Fig. 2A). A similar pattern had been observed for the GPR15358−
R18AAA and GPR153358−R18Wt (Fig. 1D). These data sug-
gested that the difference of protein sizes represents post-
translational modifications associated with the cell surface
transport. The N-terminal extracellular region of an HIV co-
receptor CCR5 is O-glycosylated on Ser and Thr residues and
this modification is required for efficient binding of a chemo-
kine ligand (23). Because GPR15 has no N-glycosylation mo-
tifs, we tested the possibility of O-glycosylation. Treatment of
Wt GPR15 protein with the enzyme mixture containing O-
glycosidase reduced the size of slower migrating band (Fig.
2B), suggesting the presence of O-linked glycans. We also
used a mutant CHO cell line ldlD to confirm this possibility.
The ldlD cells lack the UDP-galactose (Gal)/UDP-N-acetyl-
galactosamine (GalNac) 4-epimerase and therefore are defi-
cient in GalNac O-glycosylation and galactosylation in the
absence of exogeneously added GalNac and Gal, respectively
(24). When expressed in ldlD cells in the absence of GalNac and
Gal, the Wt GPR15 protein exhibited only ~40 kDa band
identical to that of GPR15358A (Fig. 2C, lane 1). The addi-
tion of both sugars in the culture restored the ~45-kDa band
for Wt GPR15 but not for GPR15359A (Fig. 2C, lanes 2 and 4).
Moreover, the GPR15 mutants in which Ser and Thr residues
at the amino acids 6, 7, 16, 17, and 20 in the N-terminal extra-
cellular region were changed to Ala showed almost complete
loss of ~45-kDa band (Fig. 2D, upper panel). This TS-AA
mutant maintained 14-3-3 binding (Fig. 2D, lower panel), sug-
gest that these N-terminal mutations at least did not sig-
nificantly affect receptor folding in the ER. Collectively, these
results suggest that GPR15 undergoes O-glycosylation on its
N-terminal Ser and/or Thr residues in the Golgi, and this
process is facilitated by 14-3-3 binding. A comparative analy-
sis of immunoprecipitated GPR15 proteins from the whole
Cell lysate and the cell surface pool shows that the O-glycosy-
lated mature form is enriched on the cell surface (Fig. 2E, up-
per panel). The presence of 14-3-3 proteins in this cell surface
IP eluate (Fig. 2E, lower panel) indicates that 14-3-3 proteins
are in complex with GPR15 proteins that were delivered to cell
surface.

To further investigate the role of 14-3-3 in the early secre-
tory pathway, we performed immunofluorescence staining in
Hela cells transfected with GPR15. The surface staining of
unpermeabilized cells (Fig. 2F) shows much higher signal of
Wt GPR15 on the plasma membrane than S359A mutant,
consistent with the FCM results (Fig. 1). When stained for the
intracellular localization in permeabilized cells (Fig. 2G), Wt
GPR15 was mostly observed in the intracellular compart-
ments that co-localized with markers for cis-Golgi (GM130)
and trans-Golgi (p230) as well as in the plasma membrane. In
contrast, S359A mutant showed less co-localization with
Golgi markers and appeared to be more co-localized with the
ER marker (calnexin) than Wt GPR15 did. Collectively, our
data support the notion that 14-3-3 binding promotes the
ER-to-Golgi trafficking of GPR15 and this leads to the en-
hanced surface expression of this receptor.

GPR15 C Terminus Contains ER Localization Signal that
Functions in the Absence of 14-3-3 Binding—How does 14-3-3
binding promote ER-to-Golgi transport of GPR15? Because a
stretch of Arg residues (RXR motif) is known to mediate
Golgi-to-ER retrograde transport of various membrane
proteins (8, 15, 25–30), we tested if the C-terminal Arg-
containing sequence of GPR15 possesses the ER localiza-
tion activity. Truncation of the last two residues (Ser359
and Leu660) substantially reduced the surface expression
(Fig. 3, A and C, GPR15358A and GPR15351) restored the effi-
cient surface expression (Fig. 3, A and C) and O-glycosylation (Fig. 3, B and C) despite the
absence of 14-3-3 binding. These results suggested that C-ter-

nal tail of GPR15 contains the ER localization activity that
functions in the absence of 14-3-3 binding. To further test
this possibility, we fused GPR15 C-terminal sequence to CD8,
a heavily O-glycosylated surface membrane protein (31). Fu-
sion of GPR15359A tail sequence (CD8-RRKKRSVSL) abol-
ished the cell surface expression, as determined by FCM (Fig.
3D) and immunochemistry (Fig. 3E, unpermeabilized cells).
The loss of surface expression in CD8-RRKKRSVSL was
consistent with its substantial co-localization with the ER
marker (Fig. 3E, permeabilized cells) and the total absence
of O-glycosylated mature form (Fig. 3F, upper panel). In con-
trast, CD8 fused with Wt GPR15 tail (CD8-RRKKRSVSL)
showed robust surface expression (Fig. 3D), less localization
in the ER (Fig. 3E), and detectable O-glycosylation (Fig. 3F,
upper panel). Importantly, this CD8 fusion showed strong
interaction with 14-3-3 (Fig. 3F, lower panel). Together, these
results demonstrate that GPR15 C terminus has the ER lo-
calization activity which functions in the absence of Ser359-de-
pendent 14-3-3 binding.

C-terminal Arg356 and Ser359 Are Most Critical for 14-3-3
Binding to GPR15—To further define the responsible residues
for 14-3-3 binding, we created more Ala mutants and exam-
ined for their surface expression and 14-3-3 binding. Other
than the Ser359, the mutation of Arg356 caused most drastic
reduction of surface expression (Fig. 4, A and B) and complete
loss of 14-3-3 association (Fig. 4C). This was consistent with
our earlier finding that the minimal component of C-terminal
mode III 14-3-3 binding motif is RXX(S/T)X-COOH (22).
The mutation of Arg354, which constitutes an Akt consensus
motif RXRXXS/T together with Arg356, showed detectable
but less effect on 14-3-3 binding. Thus, Arg356 and Ser359
seem to be the most critical components of 14-3-3 binding
activity within the C-terminal 9 amino acids of GPR15. How-
ever, the higher surface expression of S356A compared with
that of S359A in spite that both mutants completely lack 14-
3-3 binding suggests that Arg356 may be also a component of
the ER localization signal.

It is interesting to note that all of the Ala mutations in
amino acids 352–355, particularly R352A and R354A, showed
higher surface expression and more efficient O-glycosylation
than those of Wt without detectable increase of 14-3-3 bind-
ing. This is similar to the phenotype observed for the truncation
mutant Δ351 in Fig. 3. These results indicate that in the
Wt GPR15-transfected HEK293 cells, not all the GPR15 receptors are released from the ER localization pathway, probably due to the incomplete 14-3-3 binding in the basal condition. This is consistent with our previous observation that PI3K pathway activation can enhance 14-3-3 binding and surface expression of Wt GPR15 in HEK293 cells (19).

Another point to note is that the V358A mutant showed substantially higher 14-3-3 binding and surface expression than Wt did (Fig. 4). This indicates that Ala in 358 position is favored for kinase recognition and/or 14-3-3 binding. Indeed, a recent bioinformatic study shows that Ala is the most represented residue in the H11002 position relative to phospho-Ser/Thr (corresponds to Ser359 of GPR15) in all the known 14-3-3 binding sites of mammalian proteins (32).

C-terminal Arg352 and Arg354 Are Most Critical for COPI Binding and the ER Localization Activity—We then created additional mutations in the context of S359A to define the residues involved in the ER localization activity. Among those double Ala mutants, R354A/S359A and R352A/S359A showed most effective restoration of surface expression (Fig. 5A). Other mutations at Arg356, Lys355, or Arg353 in the context of S359A were less effective but still showed detectable restoration. These results indicate that Arg354 and Arg352 are most important but Arg356, Lys355, and Arg353 are also involved in the ER localization activity that is functional in the absence of 14-3-3 binding. Because an RXR motif has been reported to mediate retrograde transport by the interaction with COPI complex (14, 25, 33), we tested if GPR15 interacts with COP, an essential COPI subunit that directly recognizes cargo proteins (34). The GPR15 proteins were immunoprecipitated and blotted for COP. The C-terminal sequences of Wt or S359A mutant GPR15 were fused to Myc-CD8 protein and expressed in HEK293 cells. Histograms show cell surface expression of CD8 fusions analyzed by FCM. E, surface (unpermeabilized) and intracellular (permeabilized) localization of CD8 fusions. Hela cells transfected with Myc-CD8 fusion constructs were stained for Myc (CD8) and calnexin. Representative confocal images for CD8 (green), calnexin (red), and their colocalization (yellow). The enlarged images of selected areas (squares) were also shown. Nuclei are shown in blue. F, immunoblot for CD8 fusions and 14-3-3 association. Total lysates from cells expressing CD8 fusions were blotted for GPR15, and the immunoprecipitants were blotted for 14-3-3.

FIGURE 3. GPR15 C terminus contains ER localization signal that functions in the absence of 14-3-3 binding. A, surface expression of C-terminally truncated GPR15. C-terminally truncated HA-GPR15 mutants (Δ358, Δ354, Δ351) expressed in HEK293 cells were analyzed for surface expression by FCM. Filled histograms from GPR15-transfected cells were overlaid with that of mock-transfected cells (unfilled). The values represent mean fluorescence of the transfected cells. B, 14-3-3 association with C-terminally truncated GPR15. Truncated GPR15 proteins were immunoprecipitated and the eluates were blotted for HA and 14-3-3. C, relative surface expression levels and O-glycosylation efficiency of GPR15 truncation mutants. The mean fluorescence of each mutant from a representative FCM analysis was normalized to that of Wt GPR15 (black column). The intensity of mature and immature GPR15 bands from a representative immunoblot was measured for each construct using the molecular imager, and the mature/immature ratio was normalized to that of Wt GPR15 (gray column). D, effect of GPR15 C-terminal sequence on surface expression of CD8 fusion. The C-terminal sequences of Wt or S359A mutant GPR15 were fused to Myc-CD8 protein and expressed in HEK293 cells. Histograms show cell surface expression of CD8 fusions analyzed by FCM. E, surface (unpermeabilized) and intracellular (permeabilized) localization of CD8 fusions. Hela cells transfected with Myc-CD8 fusion constructs were stained for Myc (CD8) and calnexin. Representative confocal images for CD8 (green), calnexin (red), and their colocalization (yellow). The enlarged images of selected areas (squares) were also shown. Nuclei are shown in blue. F, immunoblot for CD8 fusions and 14-3-3 association. Total lysates from cells expressing CD8 fusions were blotted for GPR15, and the immunoprecipitants were blotted for 14-3-3.
S359A mutant was associated with more β-COP than Wt GPR15 did. It is of note that GPR15Δ351, which lacks C-terminal 9 amino acids, was associated with similar amount of β-COP to that of Wt GPR15 (Fig. 5B). This indicates that multiple regions of GPR15 are involved in the interaction with COPI. The double Ala mutants R354A/S359A and R352A/S359A showed lower level of β-COP binding than that of S359A, suggesting the critical involvement of Arg354 and Arg352 for COPI interaction. This notion is supported by the robust surface expression by these mutants (Fig. 5A).

To confirm the interaction of GPR15 with COPI, we performed an in vitro pull-down assay using the synthetic peptides described in Fig. 1B. The unphosphorylated peptide showed strong binding to β-COP and γ-COP (Fig. 5C, top and middle) but not to α-COP (data not shown). On the other hand, the peptide with phosphorylated Ser359 showed robust 14-3-3 binding but no detectable COPI binding. Moreover, the CD8-RRRKSVAL, which lacks 14-3-3 binding (see Fig. 3C) showed higher β-COP binding than CD8-RRRKSVSLL did (Fig. 5D). These findings support the mechanistic model where phosphorylation of Ser359 and subsequent 14-3-3 binding to GPR15 C terminus releases the receptor from ER retrieval/retention pathway by preventing the interaction of COPI complex with the Arg-based signal comprising the residues at 352–356 (Fig. 5E).

14-3-3 Binding Stabilizes GPR15 Protein—As shown in Figs. 4 and 5, the steady-state surface expression level of R352A mutant, which has defective RXR motif but maintains 14-3-3 binding, is higher than that of a double Ala mutant R352A/S359A that has defective RXR motif and lacks 14-3-3 binding. This suggests that the enhancing effects of 14-3-3 binding on the cell surface expression of GPR15 is not solely the suppression of ER localization activity. Because 14-3-3 proteins are known to regulate stability of various substrate proteins (35–38), we were interested in whether the 14-3-3 binding affects the protein stability of GPR15. To test this, we performed the metabolic labeling assay. Fig. 6A shows the autoradiography of [35S]Met/Cys-pulsed GPR15 proteins after indicated chase periods. The poor conversion of the immature receptor (lower arrow) into O-glycosylated mature form (upper arrow) in S359A mutant as compared with Wt and the other two mutants lacking Arg352 was consistent with the idea that the RXR signal prevents the efficient ER-to-Golgi trafficking of GPR15. The S359A showed substantially shorter half-life of about 3 h than those of Wt and R352A mutant (>5 h) (Fig. 6B). Interestingly, even though the double mutant R352A/S359A showed efficient receptor maturation due to the disturbed RXR signal (Figs. 6A and 5B), the protein stability of this mutant was as short as that of S359A (Fig. 6B). To confirm this we also examined the protein stability in the presence of cycloheximide. The rate of degradation of GPR15 protein seemed higher in the S359A and R352A/S359A mutants when compared with those of Wt.
and R352A mutant (Fig. 6C), which was consistent with the results from pulse-chase assay. These data collectively demonstrate that 14-3-3 binding increases the stability of GPR15 protein independently of the ER localization signal, and suggest that this may enhance steady-state surface expression of GPR15.

**DISCUSSION**

GPR15 is known as a co-receptor for distinct strains of HIV and SIV (17, 39). Both *in vitro* and *in vivo* studies have suggested that GPR15, in addition to its role in viral transmission, may be responsible for the enteropathies in HIV and SIV infection by inducing the intracellular signaling (40–42). Moreover, GPR15 receptor expressed on neutrophils may be involved in neutropenia that correlates with the progress of SIV (43). It is well known that the defective cell surface expression of an HIV co-receptor CCR5 results in the long-term non-progression of HIV infection (44). Hence, elucidation of molecular interactions that control cell surface density of GPR15 may provide new and important basis for understanding the pathophysiology of HIV.

Whereas the molecular mechanisms governing the forward transport of GPCRs are still less well understood when compared with the extensive studies on their ligand-induced endocytic pathways, emerging evidence suggest that ER-to-Golgi and post-Golgi trafficking of GPCRs are the highly regulated processes that involve specific conserved sorting motifs and regulatory proteins (1, 45, 46). Our results demonstrate that phosphorylation and subsequent 14-3-3 binding critically promote the surface expression of GPR15 (Fig. 1). To elucidate the underlying mechanisms, we focused on the role of 14-3-3 in regulating the ER-to-Golgi trafficking of this receptor. Ala mutants of GPR15 (Figs. 4 and 5) and the C-terminal sequence-fused CD8 (Fig. 3) revealed the ER localization signal of RXR type in the upstream of 14-3-3 binding site. The co-IP and the *in vitro* pull-down assay strongly suggest the COPI involvement in this ER localization activity (Fig. 5).

Our results collectively support the working hypothesis that
phosphorylation-dependent 14-3-3 binding to GPR15 occurs in the early secretory pathway and this suppresses the ER localization activity that is mediated by the interaction of RXR motif and COPI complex.

Promotion of cell surface transport of membrane proteins is one of the emerging roles of 14-3-3 in the recent years (12, 13). Our first result add a first example of GPCR to the growing list of such proteins. Because 14-3-3 binding often requires the AGC protein kinase recognition sequences comprising one or more Arg upstream of Ser/Thr, it is not surprising that some of those Arg residues can also serve as an ER localization signal of RXR type. Therefore, there would be conceivably more membrane proteins than currently known that utilize this system to control the cell surface density. On the other hand, 14-3-3 proteins are known to form complex with variety of molecules that are closely involved in vesicular trafficking, which suggests their scaffolding role in membrane protein transport (12). Indeed, very recent study demonstrated that 14-3-3 proteins recruit microtubule motor proteins dynein/dynactin to the cargo protein complex N-cadherin/β-catenin and thereby mediate the ER export of N-cadherin (16). These observations suggest that 14-3-3 promotes cell surface transport of membrane proteins by diverse mechanisms depending on the other molecular interactions involved in the biogenesis and trafficking of the client proteins (12, 13).

The RXR-type ER localization signals have been reported in an increasing number of surface membrane proteins (21, 25, 29, 30, 33, 47–49). In many cases the RXR motif is thought to be exposed upon incomplete folding and/or assembly, hence serving as a checkpoint for protein quality control in the early secretory pathway (15, 25, 33, 50). However, recent studies have shown that the RXR motif also functions for the constitutive ER targeting of the resident ER membrane proteins (51–53). In GPCRs, the information on the role of RXR motif in forward trafficking has been limited to GABA subreceptor. The trafficking of unassembled GABAB subunits to the plasma membrane is prevented by an RXR signal (RSRR) in the cytoplasmic tail (47, 54, 55). This RXR motif had been proposed to be shielded by C-terminal coiled-coil interaction of the GABA subunit with the GABA subunit. GABA also interacts with 14-3-3, but in this case 14-3-3 proteins do not affect the activity of RXR signal (28). Although several studies including ours (8, 14, 15, 27, 48) suggest that COPI interaction with RXR motif is interfered by 14-3-3 binding, whether the RXR motif is physically masked by 14-3-3 proteins awaits structural evidence. Interestingly, Gassmann et al. (56) have shown that the inactivation of RXR motif in GABA does not require coiled-coil interaction with GABA and suggested that it may occur by repositioning of the RXR motif into the membrane-proximal non-functional zone where the retrograde transport machineries such as COPI may not be accessible. This mechanism has been also suggested for MHC Class II-lip35 complex, where the RXR motif on the N terminus of lip35 was functionally inactivated by heterodimerization with the Class II β chain (29). This working model is strongly supported by our earlier study which revealed the functional zones for different ER localization motifs, i.e., an RXR motif is not functional when placed too close to the transmembrane region while a di-lysine (KKXX-COOH) motif remains functional (57). Therefore, 14-3-3 binding to GPR15 may either physically mask the nearby RXR motif or instead stabilize the C terminus in a distinct conformation that would place an RXR motif in the non-functional zone where its interaction with COPI may be limited.

Our kinetic analysis of degradation revealed that 14-3-3 binding increases the stability of GPR15 (Fig. 6). 14-3-3 proteins are known to control stability of various proteins (35–38). Several studies have shown that the modulation of specific protein degradation pathways is the underlying mechanism (38, 58, 59). For instance, the surface expression level of the epithelial sodium channel ENaC is positively regulated by 14-3-3. This is caused by the phosphorylation-dependent binding of 14-3-3 to the ubiquitin E3 protein ligase Nedd4–2, which leads to the inhibition of ubiquitination and degradation of ENaC (38). Hence, a shorter half-life of S359A compared with that of Wt suggests that 14-3-3 binding prevents the ER-associated degradation (ERAD) of GPR15 by suppressing the retrograde transport. However, a double mutant R352A/S359A which lacks 14-3-3 binding but efficiently delivered to cell surface due to the defective RXR motif also showed a shorter half-life than that of R352A (Fig. 6). This is involved in the biogenesis and trafficking of the client proteins (12, 13).
consistent with the lower steady-state surface expression of R352A/S359A than that of R352A (Figs. 4A and 5A). These results suggest that the stability of GPR15 protein is regulated by 14-3-3 binding independently of the RXR signal. Because this stabilizing effect of 14-3-3 binding was also observed with the GST-fusion of C-terminal 50 amino acid sequences from GPR15 (data not shown), 14-3-3 may inhibit the degradation pathway targeting the C-terminal cytoplasmic tail of this receptor. Thus 14-3-3 proteins may enhance cell surface expression of GPR15 by multiple mechanisms.

Our results suggest that phosphorylation and subsequent 14-3-3 binding to GPR15 occur in the early step of ER-to-Golgi transport and release the receptor from ER retrieval pathway. An interesting question is whether the 14-3-3 proteins remain associated with the receptor all along the trafficking to the plasma membrane. The presence of 14-3-3 signal in the immunoprecipitant of surface pool GPR15 (Fig. 2E) suggests that they are in complex with GPR15 at the plasma membrane. On the other hand, the C-terminal phosphorylation signal of GPR15 did not show enrichment of the mature form (Fig. 1F), and also our in vivo pull-down assay using GST-14-3-3 showed no enrichment of the mature form in the 14-3-3-bound fraction (data not shown). One possible explanation to reconcile these observations may be that the GPR15 proteins undergo dephosphorylation in the Golgi or during the post-Golgi transport that causes dissociation of 14-3-3 proteins, but can be phosphorylated again and bind to 14-3-3 in the later steps. The preferential phosphorylation and 14-3-3 binding to the immature form of membrane protein in the early step of biogenesis and their later reversal has been also observed in the 14-3-3-dependent surface transport of metalloproteinase ADAM22 (48). Our recent study has demonstrated the important role of PI3K pathway in promoting the 14-3-3 binding and cell surface expression of GPR15 (19). Thus, the cell surface density of GPR15 is controlled by the 14-3-3 protein binding which depends on the spacio-temporally regulated kinase and phosphatase activities. Elucidation of such pathways warrants further investigations.

The protein database shows that the potential RXR motif is present in a large number of GPCRs. For instance, more than 100 human GPCRs carry either RRR or RKR sequences in the cytoplasmic regions (data not shown). Although the actual ER localization activity of RXR motif depends on various factors such as composition of the flanking residues (60) and its relative position from the membrane (57), it is conceivable that more GPCRs than so far known utilize RXR motifs for protein quality control. Having that the GPCRs often show poor surface expression in the heterologous cells, identification and modulation of the RXR motif would be a reasonable approach to facilitate the assay development for GPCRs that await deorphanization, agonist/antagonist discovery, and functional characterization (1). The mimetic peptide that successfully inhibits the RXR motif activity in the mutant cystic fibrosis transmembrane conductance regulator (CFTRΔ508) (26) represents a promising example that supports such approach. In addition, suppression of RXR motif activity by 14-3-3 binding can be utilized for engineering of the system in which the cell surface density of the membrane proteins can be controlled by specific signaling pathways.

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