A Role for ADP-ribosylation Factor 6 in the Processing of G-protein-coupled Receptors*

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After agonist-induced internalization, the vasopressin V2 receptor (V2R) does not recycle to the plasma membrane. The ADP-ribosylation factor (ARF) proteins initiate vesicular intracellular traffic by promoting the recruitment of adaptor proteins; thus, we sought to determine whether ARF6 could promote V2R recycling. Neither the agonist-induced internalization nor the recycling of the V2R was regulated by ARF6, but a constitutively active mutant of ARF6 reduced cell-surface expression 10-fold in the absence of agonist treatment. Visualization of the ARF6 mutant-expressing cells revealed a membrane staining pattern of the V2R instead of the normal plasma-membrane expression. Analysis of V2R maturation revealed that reduced cell-surface expression was due to the diminished ability of the newly synthesized receptor to migrate from the endoplasmic reticulum to the Golgi network. The same mechanism affected processing of the V2R and acetylcholine M2 receptors. Therefore, ARF6 controls the exit of the V2R and other receptors from the endoplasmic reticulum in addition to its established role in the trafficking of plasma-membrane-derived vesicles.

The vasopressin V2 receptor (V2R) is a member of the G-protein-coupled receptor (GPCR) superfamily of receptors that are characterized by seven membrane-spanning domains (1). GPCR plasma membrane levels are maintained at a steady state by a combination of new receptor synthesis, proteolytic receptor degradation, physical sequestration from the plasma membrane, and recycling back to the plasma membrane (2). Receptor removal from the cell surface to an intracellular compartment is typically induced by the application of agonist. V2Rs have been shown to internalize in clathrin-coated pits via a process that requires both arrestin and dynamin (3). Strikingly, upon agonist removal, the V2R does not recycle back to the plasma membrane even 6 h after agonist removal (4). Regardless of the V2R expression levels in stable or transient lines or the cell line in which the receptor is expressed, the V2R still does not recycle. The receptor remains localized in an intracellular compartment, tentatively identified as the perinuclear recycling compartment, as determined by co-localization with established intracellular markers (5).

Post-translational modifications also affect the expression and distribution of V2Rs. The palmitoylation of cysteines 341 and 342 in the carboxyl terminus of the V2R, although not essential for plasma membrane delivery, results in increased receptor abundance at the plasma membrane (6). Agonist-induced phosphorylation of the V2R, exclusively by G-protein-coupled receptor kinases, resulted in receptor sequestration from the plasma membrane and trapping inside the cell (7, 5). After removal of the agonist, persistent phosphorylation accounted for the failure of the V2Rs to recycle to the plasma membrane (8). Elimination of the phosphate acceptor sites resident in the V2R carboxyl terminus imparted recycling properties to these mutant receptor species (4). Visualization of the phosphorylation-deficient V2R revealed their sorting to the same perinuclear compartment as the wild type receptor, but unlike the wild type V2R, their lower level of phosphorylation allowed them to recycle (4).

The ADP-ribosylation factor (ARF) family of proteins mediates various intracellular trafficking processes that involve membrane-bound organelles (9). ARFs are small GTPases of ~20 kDa in size that act as molecular switches. In the GDP-bound “off” state, ARFs are inactive, whereas when bound to GTP they are in the “on” state. Active, GTP-bound ARFs associate with membranes where they have effects on local lipid generation and actin rearrangement (10). In general, ARFs exert their effects on cellular trafficking by recruiting coat proteins to membranes, activating lipid-modifying enzymes and modulating the actin cytoskeletal arrangement (11). Of the six members in the ARF family, ARF1 has been the most extensively characterized to date. Whereas ARF1 is localized to the Golgi network, ARF6 was described to exclusively affect trafficking between the plasma membrane and endosomes (12). Specifically, ARF6 was shown to control a clathrin-independent recycling pathway that was dependent on the full ARF6 activation/inactivation cycle as well as phospholipid hydrolysis (13, 14). Overexpression of a GTPase-activating protein for ARF6, which catalyzed the hydrolysis of GTP to GDP thereby inactivating ARF6, reduced β2-adrenergic receptor internalization (15), highlighting the importance of ARF6 in the internalization of a GPCR.

Point mutations of ARF6 have been generated to elucidate the localization and function of ARF6 in vivo. Using ARF1 as a model, substitution of glutamine with leucine at position 67 (Q67L ARF6) resulted in an ARF6 isoform that could not hydrolyze GTP and was, thus, constitutively active (16). Furthermore, substitution of threonine with asparagine at position 27 resulted in an ARF6 that could not exchange GDP for GTP and was, thus, inactive. Even though much of the T27N ARF6 protein aggregates intracellularly, a sufficient level of T27N ARF6 localizes to the plasma membrane where it acts as a dominant-negative inhibitor of ARF6 function by sequestering nucleotide exchange factors that activate ARF6 (17). These tools have been used to examine the ARF6 dependence of cell-surface protein trafficking. It has been shown that Q67L ARF6 enhanced the rate and extent of recycling of the transferrin (Tfn) receptor as well as diminishing TfnR endocytosis (16). TfnRs have been used as models for vesicular and GPCR trafficking, and so it was interesting to determine whether ARF6 would play an analo-
gous role in the trafficking of the V$_2$R given the failure of this particular GPCR to recycle. In the absence of agonist application, Q67L ARF6 reduced cell-surface V$_2$R number by more than 10-fold, although neither the agonist-induced internalization nor the recycling of the V$_2$R was altered by the ARF6 constructs. This is contrary to what happens with the TfnR, whereby TfnR levels at the plasma membrane were enhanced 2-fold (16), but similar to the effect of Q67L ARF6 on acetylcholine M2 receptor expression (18). Thus, while attempting to delineate the route(s) of V$_2$R trafficking affected by ARF6, we found a role for ARF6 in GPCR maturation.

**MATERIALS AND METHODS**

**Reagents**—Cell culture supplies and media were obtained from Invitrogen. Tritiated AVP and 35S-Express protein labeling mix were from PerkinElmer Life Sciences. CALPHOS$^\text{TM}$ mammalian transfection kit and anti-LAMP1 monoclonal antibody were from BD Biosciences. Complete$^\text{TM}$ protease inhibitor mixture was from Roche Applied Science. The following reagents were obtained from Molecular Probes (Eugene, OR): Alexa Fluor 488-conjugated anti-HA monoclonal, Alexa Fluor 488-conjugated goat anti-mouse, Alexa Fluor 568-conjugated goat anti-rabbit, and Texas-red conjugated Tfn. Anti-endoosomal autoantigen 1 polyclonal was from Upstate Biotechnology, Inc. (Lake Placid, NY). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). Wild-type, Q67L, T27N ARF6 constructs and the ARF6-specific rabbit polyclonal antibody were generous gifts from Dr. J. G. Donaldson (National Institutes of Health, Bethesda, MD). Plasmids encoding fragment 319–418 arrestin 2 GFP and K44A dynamin were generous gifts from Dr L. Slice (University of California, Los Angeles, CA). Plasmids encoding ARNO were a generous gift from Dr. J. E. Casanova (University of Virginia at Charlottesville, VA). The plasmid encoding the phospholipase D1 pleckstrin homology domain fused to the green fluorescent protein (PLC$^\text{B1-PH-GFP}$) was a generous gift of Dr. T. Balla (NICHD, National Institutes of Health, Bethesda, MD). All other reagents were obtained from Sigma.

**Cell Culture and Transfection**—HEK 293-T cells were transiently transfected with 6 μg of plasmid DNA using the CALPHOS$^\text{TM}$ mammalian transfection kit according to the manufacturer’s instructions. After 24 h the cells were plated onto 24-well plates or 100-mm dishes and analyzed 24 h later.

**Immunoblot Analysis of V$_2$R and ARF6 Expression**—HEK 293-T cells were lysed by incubation in hypotonic buffer (50 mM Tris HCl, pH 7.5, 0.5 mM MgCl$_2$, 150 mM potassium acetate, 1% Nonidet P-40, 1.5 mM dithiothreitol) containing the Complete$^\text{TM}$ protease inhibitor mixture). Lysis was achieved by drawing the cells through needles of decreasing gauge (20–25 gauge) fitted to a 1-ml syringe. A post-nuclear supernatant was prepared by centrifugation at 3000 rpm for 5 min. After protein concentration determination, the supernatant was incubated with 2× sample buffer for 20 min. Samples were resolved by SDS-PAGE. The V$_2$R was detected using a peptide-purified rabbit polyclonal antibodies raised against a peptide corresponding to the carboxyl terminus (antibody 3) of the human V$_2$R (7). ARF6 was detected using an ARF6-specific rabbit polyclonal antibody raised against the carboxyl region of ARF6 (19). Proteins were transferred to nitrocellulose using a Trans-Blot semi-dry blotter (Bio-Rad). The nitrocellulose was blocked for 30 min in blotto (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 5% powdered milk, 0.1% Tween 20) followed by incubation with primary antibody (1:1000) overnight at 4 °C. The blot was washed 3 times for 10 min in blotto and incubated with horseradish peroxidase-coupled secondary antibody) for 1 h at room temperature. After 3 more 10-min washes in blotto, the proteins were visualized by ECL.

**Measurement of Receptor Binding**—Transfected cells were plated onto 24-well plates that had been previously coated with poly-L-lysine (100 μg/well). The cells were washed twice with ice-cold PBS and incubated with 20 nM [3H]AVP in PBS with 2% bovine serum albumin, 0.5 mM CaCl$_2$, and 2 mM MgCl$_2$. After a 2-h incubation in the cold room, the binding mixture was removed by aspiration, the cells were rinsed twice with ice-cold PBS, and 0.5 ml of 0.1 M NaOH was added to extract radioactivity. After 30 min at 37 °C, the fluid from each well was transferred to a scintillation vial containing 3.5 ml of scintillation fluid. Non-specific binding was determined under the same conditions in the presence of 20 μM unlabeled AVP. Each experimental point was assayed in triplicate. Data are presented as the mean ± S.E. of 3–5 experiments.

**Flow Cytometry**—V$_2$R-expressing HEK 293-T cells stained with an Alexa 488-conjugated anti-HA antibody (1:100) were examined on a FACSsort flow cytometer (BD Biosciences) in the presence of propidium iodide to exclude cells that had lost their membrane integrity. Cells were excited at 488 nm, and the Alexa 488 and propidium iodide fluorescence were detected at 530 and >650 nm, respectively. Ten thousand viable cells were examined per sample and analyzed using CellQuest software. An analysis gate was set on the control cells to determine the number of cells that exhibited a decrease in V$_2$R expression.

**Immunofluorescence and Confocal Laser-scanning Microscopy**—Transfected cells were seeded on glass coverslips, and if necessary, the requisite intracellular markers were added 24 h later. The cells were then incubated at 37 °C followed by 2 washes with 37 °C PBS. Cells were fixed with 4% paraformaldehyde at 4 °C for 1 h followed by 3 washes for 10 min with PBS. The cells were then permeabilized with a solution of PBS containing 0.1% saponin and 0.2% gelatin. After incubation of the cells with the primary antibody for 2 h at room temperature, the cells were washed 3 times for 10 min with PBS. The cells were then incubated with the secondary antibodies for 90 min at room temperature followed by three 10-min washes with PBS. The coverslips were mounted on microscope slides in VectorShield$^\text{TM}$ mounting medium. Cells were then visualized at 22 °C using a Zeiss LSM 510 UV confocal laser-scanning microscope using an oil 100× objective, numerical aperture of 1 (Carl Zeiss, Inc.). The 488- and 543-nm line from the included argon ion laser were the excitation sources, and a 505–550-nm band-pass filter or 560-nm long-pass filter, respectively, were used for the emission. The software used for acquisition was Zeiss LSM510 Version 3.2 for Windows 2000, and for analysis, LSM Image Examiner (licensed) Version 3.2 for Windows 2000.

**Metabolic Labeling with [35S]Methionine/Cysteine and Immunoprecipitation of GPCRs**—Proteins were labeled in 100-mm dishes using a modification of the method published by Keefer and Limbird (20) as follows. 48 h after transfection the cells were incubated for 1 h at 37 °C in methionine/cysteine-free Dulbecco’s modified Eagle’s medium. 100 μCi of 35S-Express protein-labeling mix was then added to each plate for 30 min at 37 °C (pulse). The medium was aspirated, and the cells were washed with 37 °C PBS and returned to the incubator for the stated intervals (chase). The cells were rinsed and harvested in PBS, and the cell pellet from each plate was homogenized in 500 μl of RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS containing the Complete$^\text{TM}$ protease inhibitor mixture) by drawing the cells through needles of decreasing gauge (20–25 gauge) fitted to a 1-ml syringe. Cell extracts were clarified by mixing with 50 μl of a 50% slurry of pre-washed protein A-Sepharose. Pre-washed protein A-Sepharose was prepared by incubating the resin with 100 mg/ml bovine serum albumin in RIPA buffer for 1 h followed by 2 washes with RIPA buffer alone. The clarified extracts were then incubated overnight at 4 °C with a monoclonal 12CA5 anti-HA antibody (21). Antigen-antibody complexes were then separated by incu-
bating the mixture with pre-washed protein A-Sepharose for 2 h at 4 °C. The beads were centrifuged, incubated 4 times for 4 min at room temperature with RIPA buffer, and recovered each time by centrifugation. Proteins were eluted with 80 μl of 2× Laemmli buffer containing 10% β-mercaptoethanol. The samples were electrophoresed in 10% polyacrylamide gels and visualized by exposing the dried gels to Eastman Kodak Co. BioMax film at -70 °C. Densitometric analysis of the labeled bands was carried out using a ChemiDoc XRS gel-imaging system (Bio-Rad).

RESULTS

Constitutively Active ARF6 Inhibits Cell-surface V2R Expression—We have previously shown that the mature wild-type V2R migrates as a diffuse band of 45–55 kDa on SDS-PAGE gels (21). This migratory pattern arises from both N- and O-linked glycosylation, which takes place in the endoplasmic reticulum and Golgi apparatus (22). A V2R mutant in which asparagine at position 22 was changed to glutamine (N22Q V2R) lacks N-linked glycosylation and migrates as a focused band of 40 kDa. As previously reported, this receptor form is unaltered in its ligand binding affinity, second-messenger generation, and trafficking patterns (21). As a result, the N22Q V2R was used as the wild-type V2R for this study to obtain sharper, better-resolved bands during protein electrophoresis.

Published reports describing a role for ARF6 in the recycling of internalized endosomes to the plasma membrane led us to examine whether this small GTPase could promote the recycling of the V2R. For this purpose, cDNAs encoding wild-type, constitutively active (Q67L), or dominant-negative (T27N) forms of ARF6 were co-transfected with V2Rs in HEK 293-T cells. In the absence of any agonist treatment, co-expression of the V2R with Q67L ARF6 reduced the cell-surface levels of receptor by greater than 90% (Fig. 1A, left y axis), as detected by radio-ligand binding. After a 20-min incubation with a saturating concentration of agonist (100 nM AVP), neither the extent of endocytosis nor lack of recycling of the V2R was affected by expression of either T27N or Q67L ARF6 (data not shown). More modest reductions in V2R surface expression were observed when the V2R was co-expressed with either wild-type or T27N ARF6 (Fig. 1A). In the absence of any agonist treatment, co-expression of the V2R with either the wild-type or T27N (GDP-bound and, thus, inactive) ARF6 reduced surface receptors in whole cells by 42 ± 2 and 22 ± 8%, respectively. Both levels were significantly different (n = 3, p < 0.01, one-way analysis of variance, Dunnett’s post-test) from control (that is, those transfected with the V2R and β-galactosidase (β-gal). This Q67L-mediated effect was not restricted to the Gs-coupled V2R, as the recycling-competent Gi-coupled V1aR was subject to similar regulation (Fig. 1A, left y axis). A similar ARF6 effect on the acetylcholine M2 muscarinic receptor cell-surface expression has been reported (18). Support for these results was obtained using fluorescence-assisted cell sorting analysis (Fig. 1A, right y axis). Here too, the proportion of viable cells showing fluorescence eminating from Alexa 488-labeled V2Rs was indistinguishable between the wild-type and T27N-transfected HEK 293-T cells. However, a clear difference was observed in the fluorescence intensity of the Q67L-expressing cells, which showed 33% of the intensity of control cells (Fig. 1A, right y axis).

To examine whether ARF6 modified V2R synthesis, HEK 293-T cells transfected with the V2R plus the ARF6 constructs were lysed by hypotonic buffer, as described under “Materials and Methods,” and the aqueous extract obtained from this process was immunoblotted for the presence of the V2R (Fig. 1B) or ARF6 (Fig. 1C). Overexpression of the ARF6 constructs resulted in an ~10-fold increase in ARF6 expression over control, untransfected cells. For the V2R, the mature 40-kDa form (arrow) and the 33-kDa precursor form (arrowhead) were detected in the cells co-transfected with the ARF6 constructs. As can be seen, there was a decrease in mature V2R expression in the presence of Q67L ARF6. The mature V2R form is the one responsible for [3H]AVP binding, and so reduced expression would have accounted for diminished ligand binding. Thus, the immunoblotting data were consistent with those obtained via radio-ligand binding and fluorescence-assisted cell sorting analysis. In the presence of Q67L ARF6, it was clear that there was a prevalence of the precursor form of the V2R and concomitant depletion of the mature form. This was in contrast to the other lanes whereby there were approximately equal levels of V2R expression in either form. Therefore, it appeared that the constitutively active Q67L ARF6 reduced the amount of mature V2R. These results were contrary to what happened with the prototypical model of recycling membrane proteins, the TfnR. The net effect observed was an increase of TfnR present at the plasma membrane unlike what we have observed (16). Thus, we attempted to delineate the route(s) for this trafficking phenomenon.
Intracellular Localization of the V2R in the Presence of Q67L ARF6—To determine whether the overexpressed V2Rs and ARFs interacted in vivo, co-immunoprecipitation experiments were carried out as described under “Materials and Methods.” As shown in Fig. 2A, immunoprecipitation of the V2R gave the previously observed pattern of V2R expression whereby the Q67L ARF6 drastically inhibited V2R expression. When parallel samples were immunoblotted for the presence ARF6 (Fig. 2B), only the Q67L ARF6 mutant was found to be associated with the V2R. The association was stable for the duration of the immunoprecipitation as it was unnecessary to add cross-linkers to recover ARF6 Q67L from the V2R precipitate. As expected, visualization of the V2R when co-transfected with either wild-type or T27N ARF6 showed a predominantly plasma-membrane distribution (Fig. 3, A and G, respectively). Endogenous ARF6 as well as overexpressed wild-type and Q67L ARF6 have been reported to have both an intracellular and plasma membrane distribution (12). In the presence of the Q67L ARF6 (Fig. 3E), the V2R was observed in “vacuolar” structures (Fig. 3D) that were distributed in distinct cytoplasmic areas. These vacuolar bodies also contained the overexpressed Q67L ARF6, as evidenced by the yellow regions on the merged image (Fig. 3F). This vacuolar pattern induced by Q67L ARF6 has been observed as early as 18 h after transfection in Cos and HeLa cells (14) and persisted for at least 48 h after transfection in HEK 293-T cells. By virtue of residing in the same compartment, this result lent support to the co-immunoprecipitation studies that had indicated an interaction between the V2R and Q67L ARF6.

To determine the nature of these vacuoles, co-localization studies using established intracellular markers were carried out. Intracellular Tfn accumulation (Fig. 4, B and E) was used to identify early and recycling endosomes (23). Tfn uptake was efficient in untransfected cells as well as in those only expressing the V2R and β-gal (Fig. 4C). Cells co-expressing the V2R and Q67L ARF6 did not show any Tfn endocytosis (Fig. 4F). Neighboring untransfected cells internalized the Tfn, indicating that the Q67L ARF6 construct functioned as previously reported (16). Other intracellular markers were used to identify localization of the V2R (Fig. 5, A, D, G, and J); that is, early endosomal autoantigen 1.
**FIGURE 5. Visualization of Q67L ARF6 vacuoles and intracellular organelle markers.** HEK 293-T cells co-expressing either the V2R and β-gal or the V2R and Q67L ARF6 were fixed and stained for endosomal autoantigen 1 (EEA1) (B and E at 1:100 dilution) or LAMP1 (H and K at 1:100 dilution). Alexa 568 anti-rabbit polyclonal (1:100) was used as the secondary antibody. The V2R-containing “vacuoles” did not co-localize with either endosomal autoantigen 1 (EEA1) (Fig. 5, B and E) or LAMP1 (Fig. 5, H and K), ruling out their identity as early/recycling endosomes or late endosomes/lysosomes. The lack of co-localization with recycling endosomes indicated that the V2R did not reside in a compartment from where it could be recycled to the cell surface.

**Q67L ARF6 Effect Is Independent of Constitutive V2R Endocytosis.** The effect of the Q67L ARF6 mutant on V2R cell-surface expression showed a concentration-effect relationship as shown in Fig. 6A. Increasing amounts of transfected Q67L ARF6 DNA (and, thus, expressed protein) resulted in a proportional decrease in V2R plasma-membrane expression (n = 6, r² = 0.81), highlighting the specificity of this effect. Furthermore, expression of ARF nucleotide binding site opener (ARNO), a GTPase exchange factor for the ARF family, with the wild-type ARF6 mimicked the reduction in cell-surface V2R number induced by the Q67L ARF6 construct (n = 4, p < 0.001) (Fig. 6B). ARNO was, thus, able to activate the overexpressed wild-type ARF6, thereby producing a similar “phenotype” to that observed with the Q67L ARF6. When co-expressed with only the V2R, ARNO expression did not affect cell-surface expression nor the agonist-induced internalization or the recycling of the V2R (data not shown). ARNO expression thereby mimics Q67L ARF6 action only when overexpressed with the wild-type ARF6. These results indicated that ARF6 activation was able to regulate V2R expression.

Actin rearrangement participates in the movement of endosomes in cells as well as being the mechanism by which ARF proteins partly exert their influence on intracellular trafficking patterns (11). These effects have been described mostly in HeLa cells that present a characteristic pattern of fibers upon actin-phalloidin staining. Contrary to these reports, actin fibers were not detected when HEK 293 cells were subjected to similar staining. Instead, the staining indicates a diffuse distribution of actin (supplemental Images 3 and 4). This result is similar to the actin distribution in resting HEK cells reported by Fujino et al. (24) and as shown in the supplemental material). Whether actin was involved in the Q67L-mediated effect was tested by disrupting actin assembly in transfected HEK 293-T cells, as shown in Fig. 7A. The cell-permeant actin disrupter, cytochalasin D, did not alter the abundance of V2R binding sites on the plasma membrane as the binding values were barely changed from 4189 ± 970 to 4356 ± 834 cpm/well (n = 4–7, p > 0.05). Thus, the effect of Q67L ARF6 was independent of cytoskeletal rearrangement.

There was a possibility that enhanced constitutive endocytosis of the V2R accounted for the Q67L ARF6 action. GPCRs mostly use the clathrin-coated pit pathway for both agonist-dependent and -independent endocytosis. Disruptors of this pathway were then used to determine whether these pathways were involved in the Q67L ARF6 effect on V2R expression (Fig. 7B). We have previously shown that the carboxyl terminus dominant-negative arrestin (319–418) mutant inhibited agonist-induced internalization of the V2R (3). However, co-expression of the mutant arrestin did not restore V2R expression levels as the binding remained low at 2490 ± 688 from 2026 ± 341 cpm/well. This was not
surprising as arrestin is generally recruited only after receptor activation by agonist application, which is absent in this case. The GTP binding-deficient dynamin (dynamin K44A), an inhibitor of the scission of clathrin-coated pits and caveolae from the plasma membrane (25), had no significant effect on the Q67L-mediated reduction in V2R surface number when co-expressed (n = 3, p > 0.05). Unlike the mutant 319–418 arrestin, the K44A dynamin mutant does not require receptor activation to function, a matter of significance as these experiments were conducted in the absence of agonist. Once again, despite the presence of the MetSO2, cytochalasin D (Fig. 7A), or the clathrin-coated pit inhibitors (Fig. 7B), surface expression of the V2R was significantly diminished in the presence of Q67L ARF6. Visualization of the V2R localized to the vacuolar structures (Figs. 8, D and J) showed no co-localization of either arrestin 2 or the α-adaptin subunit of AP2 (Figs. 8, E and K, respectively). Both arrestin 2 (Fig. 8, B and E) and AP2 (Fig. 8, H and K) are markers for GPCR-residing compartments derived from clathrin-coated vesicles (26). Therefore, the compartment in which the V2R resided under the influence of Q67L ARF6 did not arise from trafficking from the plasma membrane, a process in which ARF6 has been implicated.

**Q67L ARF6 Blocks V2R Traffic from the Endoplasmic Reticulum**—Fig. 1B showed that in the presence of Q67L ARF6, there was a depletion of mature V2Rs as detected by Western blotting. The addition of sugars in the Golgi network is responsible for the generation of two distinct N22Q-V2R isoforms that we have previously shown to migrate as a 40-kDa band (mature glycosylated form) and a 33-kDa band (immature form before the addition of O-linked sugars) (22). These V2Rs corresponded to those bands marked by an arrow and an arrowhead, respectively, in Figs. 18 and 9. Because endocytosis did not account for diminished V2R expression, altered synthesis and delivery of V2Rs was another trafficking pathway that could have accounted for this behavior. To investigate this possibility, metabolic labeling of the V2R was carried out to observe the fate of de novo synthesized receptor protein (Fig. 9A). In these experiments, cells transfected with cDNAs encoding V2R and wild-type (lanes 2–4) or Q67L ARF6 (lanes 5–7) were incubated with [35S]cysteine/methionine, and the incorporation of radioactivity into V2Rs was monitored by immunoprecipitating the labeled protein. The lane marked 0 represented the amounts of labeled mature (arrow) and precursor (arrowhead) V2R immunoprecipitated after a 30-min incubation with [35S]cysteine/methionine. The fate of the newly synthesized V2R forms was followed through time (2 and 4 h), denoted as 2 and 4, respectively. As shown in Fig. 9A, untransfected control HEK 293-T cells did not synthesize any V2Rs. The equivalent amounts of initial precursor V2Rs at time 0 indicate that the V2R was synthesized equally well in the presence of either wild-type or Q67L ARF6 (second versus fifth lanes). Under wild-type ARF6 co-expression, the immature V2R rapidly disappeared, concomitant with the progressive increase in mature V2R levels (second to fourth lanes). However, in the presence of Q67L ARF6, the immature form of the receptor decreased slowly, whereas the mature receptor barely increased from time 0 through 4 h (fifth to seventh lanes). These data suggested accumulation of the precursor protein in the endoplasmic reticulum. Calnexin is a resident endoplasmic reticulum protein that binds N-linked glycans on newly synthesized glycoproteins (27) and acts as a chaperone in the maturation of the V2R (28). Co-staining of the V2R and β-gal-transfected cells
(Fig. 10A) showed a punctate calnexin distribution in the HEK 293-T cells (Fig. 10B). However, in the presence of Q67L ARF6 (Fig. 10E), calnexin was localized along the vacuolar rings in which the V2R resided (Fig. 10D). In the presence of Q67L ARF6, the dramatic co-localization of the V2R and calnexin was evident by the regions of yellow rings in the merged image (Fig. 10F). This result indicated that the V2R was resident in a compartment that was predominantly associated with immature proteins (Fig. 10F). Thus, diminished V2R exit from the endoplasmic reticulum accounted for the reduced V2R expression observed under the influence of Q67L ARF6. Clearly in this case, ARF6 acted by controlling sorting to the Golgi network from the endoplasmic reticulum. The action of ARF6 was not restricted to the V2R as the M2-muscarinic receptor was subject to the same maturation process as the V2R (Fig. 9B).

Enhanced degradation of the V2R may have accounted for diminished cell-surface expression in the presence of Q67L ARF6, although Fig. 5F did not reveal co-localization between the lysosomal marker LAMP1 and the V2Rs. Another intracellular organelle associated with V2R degradation is the 26 S proteasome (29). However, the proportions of immature 33-kDa V2Rs were not altered by the presence of a cell-permeant proteasomal inhibitor (10 \text{\mu M} MG132), nor was there an increase in the expression of mature V2Rs under inhibition of the 26 S proteasome, indicating that neither form was degraded (Fig. 9C). This result was duplicated with another proteasomal inhibitor (10 \text{\mu M} epoxomicin,

**FIGURE 9.** Metabolic labeling and immunoprecipitation (IP) of V2R and M2-muscarinic receptors. HEK 293-T cells co-transfected with either the V2R and wild-type (WT) ARF6 or Q67L ARF6 (A) or M2-muscarinic receptor (M2R) and wild-type ARF6 or Q67L ARF6 (B) were metabolically labeled with [35S]cysteine/methionine as described under “Materials and Methods.” In both cases the first lane contains proteins precipitated from untransfected cells. The second through fourth lanes contain wild-type ARF6-transfected cells, and the fifth through seventh lanes contain the Q67L-transfected cells. 0 indicates a pulse of 30 min with [35S]cysteine/methionine and the second and fourth lanes indicate a chase of 2 and 4 h, respectively, after removal of the radioactive amino acids. The levels of receptor delivery and expression were followed through time (0, 2, and 4 h) by determining the processing of the [35S]-labeled V2Rs and M2-muscarinic receptors. Molecular weight markers are shown on the right. A and C are representative gels from experiments performed at least three times. Inset, relative intensities of the radio labeled bands derived from the precursor V2R in the presence of wild-type ARF6 (□), precursor V2R in the presence of Q67L ARF6 (○), mature V2R in the presence of wild-type ARF6 (□), and mature V2R in the presence of Q67L ARF6 (○). C. HEK 293-T cells (HEK) or those co-transfected with the V2R and Q67L ARF6 were treated with either Me2SO or the proteasomal inhibitor MG132 (10 \text{\mu M} for 2 h in cysteine/methionine-free medium) before incubation with [35S]cysteine/methionine. The synthesized V2R were labeled and immunoprecipitated as described under “Materials and Methods.”
data not shown) as well as the lysosomal inhibitor (200 μM chloroquine, data not shown). Therefore, inadequate V$_2$R processing rather than degradation accounted for the Q67L ARF6-mediated reduction in plasma membrane V$_2$R expression.

**DISCUSSION**

Nephrogenic diabetes insipidus can arise from V$_2$Rs that are trapped intracellularly and fail to migrate to the cell surface (28). For some of these aberrant V$_2$Rs, membrane-permeant inverse agonists have been shown to restore the formation of correctly folded V$_2$Rs and their resultant delivery of V$_2$Rs to the cell surface (30). These pharmacological chaperones have been useful in attempting to delineate the mechanisms through which V$_2$Rs are sorted to the plasma membrane after synthesis in the endoplasmic reticulum. Here we shed further light on this aspect of V$_2$R trafficking by describing a novel role for ARF6 in the receptor maturation and delivery to the Golgi network. To date, most of the work carried out on the functions of ARF6 have focused on trafficking occurring at the plasma membrane via clathrin-independent endosomes, particularly in the recycling of cargo protein to the plasma membrane. The data presented here demonstrate a previously unappreciated facet of ARF6-mediated membrane trafficking; namely, the proper processing and delivery of GPCRs from the endoplasmic reticulum to the Golgi network. Because the Q67L ARF6-mediated reduction in cell-surface expression was observed in three different receptor subtypes, this phenomenon may applicable to the wider GPCR family.

Similarities between the V$_2$R and the M$_1$-muscarinic receptor include endocytosis via a clathrin-dependent pathway and slow receptor recycling back to the plasma membrane upon agonist removal (31). Previous experiments have shown that the M$_1$-muscarinic receptor underwent agonist-induced endocytosis via an ARF6-dependent pathway (18). In contrast to both the β$_2$-adrenergic (32) and M$_1$-muscarinic receptors, neither the agonist-induced internalization nor the recycling of the V$_2$R was regulated by the ARF6. Experiments using flow cytometry detected a marked decline in the fluorescence intensity of Alexa 488-labeled V$_2$Rs, consistent with diminished expression. Co-expression of wild-type, Q67L, or T27N ARF6 and the M$_1$-muscarinic receptor has been carried out, and the cell-surface receptor expression was determined by radioligand binding using the cell-impermeant antagonist N-[1$^\text{H}$]methyl-

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**FIGURE 10. Co-localization of V$_2$Rs and calnexin.** HEK 293-T cells co-expressing either the V$_2$R and β-gal or the V$_2$R and Q67L ARF6 were fixed and stained as previously described under “Materials and Methods.” The V$_2$Rs (A and D) were visualized using an Alexa 488-conjugated anti-HA antibody (1:100). Calnexin (B and E) was visualized using a primary polyclonal rabbit antibody (1:1000) and an Alexa 568-conjugated secondary anti-rabbit polyclonal antibody (1:100). The respective overlays (C and F) are shown. Scale bar, 10 μm.

ARF6 Contribution to GPCR Maturation

Visualizations of the intracellular distribution of the V$_2$R, when co-expressed with the Q67L ARF6, showed an intense vacuolar-staining pattern. Similar vacuoles have been observed before in Cos and HeLa cells overexpressing Q67L ARF6 (14). We too have observed vacuolar formation as early as 24 h after transfection of Q67L ARF6 with concomitant reduced V$_2$R expression as measured by metabolic labeling (data not shown). The actin- and phosphatidylinositol 4,5-bisphosphate coated vacuolar structures seen in HeLa cells were formed by the fusion of smaller vesicles. In this case, the addition of cytochalasin D slowed down the fusion of vesicles and, thus, halted the formation of these vacuoles. As mentioned earlier, similar vesicles cannot be detected in HEK 293 cells. In HEK cells the addition of cytochalasin D did not prevent the loss of V$_2$R sites as measured by radioligand binding experiments. Cytochalasin D has been shown to be an efficient inhibitor of ARF6-and actin-mediated trafficking processes such as membrane ruffling and membrane protrusion in HeLa cells (13). In CHO cells, ARF6 did not co-fractionate with clathrin-derived vesicles (33), indicating that they were neither endosomal nor lysosomal compartments. Visualization of the V$_2$R in the presence of Q67L ARF6 also showed an absence of staining for endosomes, lysosomes, or other clathrin-coated pit-derived compartments but did show co-localization with calnexin. Consequently, these results identified two distinct ARF6-mediated processes that were altered by the Q67L mutant protein; 1) an actin-dependent process whereby an intact actin cytoskeleton was required for the efficient recycling of membrane back to the cell surface in CHO cells (failure of this process led to the formation of the actin-coated vacuoles) and 2) an actin-independent mechanism that accumulated intracellular V$_2$Rs in HEK 293-T cells, which resulted in V$_2$Rs clustering in intracellular calnexin-associated vacuolar structures, consistent with an inhibition of trafficking of the V$_2$R from endoplasmic reticulum. The latter observation accounted for the lack of co-localization of the V$_2$R and Golgi markers, such as TGN38 and adaptor protein 1 (data not shown), as the V$_2$R was unable to leave the endoplasmic reticulum en route to the Golgi apparatus and, subsequently, the plasma membrane.

The main trafficking pathways for GPCRs in cells are internalization, recycling, and new receptor synthesis. Constitutive V$_2$R endocytosis as a factor for the Q67L ARF6 effect was excluded through the use of the dominant-negative clathrin-coated pit inhibitors. The failure to enhance V$_2$R expression by the dominant-negative dynamin coupled with the lack of co-localization with clathrin-coated pit markers indicated Q67L ARF6 did not affect constitutive endocytosis. Due to the absence of V$_2$R internalization, V$_2$R recycling was not likely to occur as there were few, if any, V$_2$Rs localized in the recycling endosomes. Further support for the exclusion of these processes was provided by the absence of co-localization between the V$_2$R and the various markers for endosomes used in the current study. Lysosomal and proteasomal inhibitors also failed to enhance mature V$_2$R expression; thus, proteolysis too could be discounted. After arrestin-dependent ubiquitinylation, V$_2$Rs are degraded by the 26 S proteasome, a process inhibited by 10 μM MG132 (29). This pharmacological treatment did not affect the levels of immature or mature V$_2$Rs expressed nor did a lysosomal inhibitor (200 μM chloroquine). Because arrestin-mediated ubiquitinylation was a prelude for V$_2$R degradation, we did not observe any effects of ARF6 expression on V$_2$R degradation in HEK 293-T cells either by pulse-chase experiments or co-localization studies with lysosomal markers. However, closer examination of the receptor synthesis process revealed a
malfunction in the maturation and delivery of the V2R to the cell surface.

It has been shown that there are no particular sequences in the carboxyl terminus of the V2R that are responsible for endoplasmic reticulum quality control and retention (34). Rather, the native conformation of the V2R was the determinant in sorting to the cell surface. However, this process is still poorly defined, and the proteins and/or chaperones involved have not been fully identified. What is known, however, is that for particular V2R mutants, chemical chaperones such as glycerol exert a folding-promoting effect by stabilizing the appropriate receptor conformation (30). Consequently, due to the reduction in cell-surface V2R expression mediated by Q67L ARF6, we envisage the scenario whereby ARF6 directly controls V2R maturation and delivery. This idea was borne from the observation that constitutively active ARF6 inhibited the trafficking of the V2R to the plasma membrane, as evidenced by metabolic labeling of the receptor and immunofluorescence experiments. In the absence of ARF6 GTP hydrolysis, immature V2Rs could not proceed beyond the endoplasmic reticulum and were, thus, shuttled to an as yet unidentified vacuolar compartment.

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