Translocation of Activator of G-protein Signaling 3 to the Golgi Apparatus in Response to Receptor Activation and Its Effect on the trans-Golgi Network*

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Background: The AGS3-Gαi complex is regulated by a GPCR, but downstream signaling events are unknown.

Results: Upon receptor activation, AGS3 translocates to the Golgi apparatus, where it regulates events at the TGN.

Conclusion: The AGS3-Gαi complex serves as a signal transducer for GPCRs.

Significance: The regulated translocation of AGS3 offers unexpected mechanisms for modulating protein secretion and/or endosome recycling events at the TGN.

Group II activators of G-protein signaling play diverse functional roles through their interaction with Gαs, Gαi, and Gαo, via a G-protein regulatory (GPR) motif that serves as a docking site for Gαi-GDP. We recently reported the regulation of the AGS3-Gαi signaling module by a cell surface, seven-transmembrane receptor. Upon receptor activation, AGS3 reversibly dissociates from the cell cortex, suggesting that it may function as a signal transducer with downstream signaling implications, and this question is addressed in the current report. In HEK-293 and COS-7 cells expressing the α2AR antagonist rauwolscine. The TPR domain of AGS3 was required for agonist-induced translocation of AGS3 from the cell cortex to the GA, and the translocation was blocked by pertussis toxin pretreatment or by the phospholipase Cβ inhibitor U73122. Agonist-induced translocation of AGS3 to the GA altered the functional organization and protein sorting at the trans-Golgi network. The regulated movement of AGS3 between the cell cortex and the GA offers unexpected mechanisms for modulating protein secretion and/or endosome recycling events at the trans-Golgi network.

Activators of G-protein signaling (AGS)3 and regulators of G-protein signaling (RGS) proteins were both discovered in functional screens for G-protein signaling modulators and essentially define a panel of biological regulators that influence signal transfer from receptor to G-protein, guanine nucleotide binding and hydrolysis and/or G-protein subunit interactions and/or serve as alternative binding partners for Gα and Gβγ independent of the classical heterotrimeric Gαiβγ. AGS and RGS proteins have revealed unexpected functional diversity for the “G-switch” signaling mechanism, expanding the functional roles of G-protein subunits and perhaps identifying new opportunities for therapeutic manipulation of G-protein signaling (1–6). RGS proteins fall into several subgroups, and there are more than 30 distinct proteins that contain an RGS or an RGS-like domain in mammalian cells (2). AGS proteins generally fall into three subgroups (1, 5). Group I AGS proteins and related entities, such as Ric-8A, GIV (Girdin), and the Saccharomyces cerevisiae protein Arr4, act as non-receptor guanine nucleotide exchange factors for Gαi and/or Gαsβγ (7–9). Group III AGS proteins interact with Gβγ or perhaps heterotrimer (1, 10). Sato et al. (11) also recently identified three transcription factors as AGS11, -12, and -13 with apparent selectivity for Gαi1α2, however, the biochemical properties of the G-protein regulation have not yet been fully characterized, and thus it is difficult to place these three AGS proteins into Group I, II, or III.

Group II AGS proteins are defined by the presence of one or more guanine nucleotide regulatory (GPR) motifs (12), also known as GoLoco or LGN motifs (13, 14), that bind Go-GDP free of Gβγ. Group II AGS proteins consist of seven proteins (AGS3 (GPSM1), LGN (GPSM2, AGS5), AGS4 (GPSM3), RGS12 (AGS6), Rap1Gap (transcript variant 1), RGS14, and PCP2/L7), each of which contains 1–4 GPR motifs for docking of Ga serving as alternative binding partners for specific subtypes of Ga. There are three types of Group II AGS proteins that are distinguished by the number of GPR motifs and/or the presence of defined regulatory protein domains (1, 5). AGS3 and LGN (AGS5) have four GPR motifs downstream of a tetra-ricopeptide repeat domain, whereas RGS12 (AGS6), RGS14, and Rap1GAP have one GPR motif plus other defined domains that act to accelerate Ga-GTP hydrolysis (1, 5).

The GPR-Gα complex is involved in an increasingly interesting set of regulatory functions that we are just beginning to understand, including roles in cell division, neuronal plastic-
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ity, autophagy, protein trafficking, hearing, morphogenesis, and the renal response to injury (15–30). Although the majority of Ga within the cell probably exists as part of the GaβY heterotrimer at the cell cortex, a subpopulation of Gaα is apparently complexed with GPR proteins. This GPR-Gα signaling module is regulated by non-receptor guanine nucleotide exchange factors, such as Ric-8A and GIV (24, 31–33), with the latter entity processing signals from cell surface growth factor receptors (24). The GPR-Gα complex (AGS3-Gαα↓, AGS4-Gα↓, RGS14-Ga↓) at the cell cortex is also regulated by a subgroup of G-protein-coupled receptors (34–36). Upon receptor activation, AGS3 reversibly dissociated from Gaα at the cell cortex (34), but the trafficking of AGS3 and its regulation following receptor activation in this system is not known. In this work, we report that AGS3 reversibly translocates from the cell cortex to the Golgi apparatus (GA), and this is associated with altered functional integrity of the trans-Golgi network. The regulated translocation of AGS3 between the cell cortex and the GA offers unexpected mechanisms for modulating protein secretion, endosome recycling, and Golgi dynamics.

EXPERIMENTAL PROCEDURES

Materials—UK-14304, U0126, and pertussis toxin were obtained from Sigma. Rauwolscine hydrochloride was obtained from Carl Roth GmbH (Karlsruhe, Germany). Polyethyleneimine (PEI) was obtained from Polysciences, Inc. (Warrington, PA). All cell culture materials were obtained from Invitrogen. HEK-293 and COS-7 cells (CRL-1573 and CRL-1651, respectively) were obtained from American Type Culture Collection (Manassas, VA). Benzyl-coelenterazine was obtained from NanoLight Technology. 96-well gray OptiPlates were obtained from PerkinElmer Life Sciences. GM130 antibody (610823) was obtained from BD Biosciences. TGN46 antibody (110-40769) was obtained from Novus Biologicals. TGN46 antibody (110-40769) was obtained from BD Biosciences. AGS3 antisera were generated in the laboratory of Dr. Jennifer Lippincott-Schwartz (37). Mannosidase II antibody (ab25631) and LAMP2 antibody (ab25631) were obtained from Abcam (Cambridge, MA). AGS3 antisera were generated in the laboratory of Dr. Dzwokai Zach Ma (University of California, Santa Barbara, CA) by immunization of rabbits with a glutathione S-transferase (GST)-AGS3 fusion protein encoded by the GPR domain (Ala467–Ser656) of AGS3 (26). Sialyltransferase-EGFP (ST-GFP) was generated in the laboratory of Dr. Jennifer Lippincott-Schwartz (37). All other reagents and materials were obtained as described elsewhere (34).

Cell Culture—HEK-293 and COS-7 cells were maintained in DMEM high glucose medium supplemented with 5% fetal bovine serum, respectively, in the presence of 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified incubator in the presence of 5% CO₂ at 37 °C. Cells were transfected using PEI as described previously (34).

Real-time Bioluminescence Resonance Energy Transfer (BRET) Measurements—HEK-293 cells were seeded on 6-well plates and cultured overnight at 37 °C. Cells were transiently transfected with donor (phRluc::AGS3), acceptor (pcDNA3::Goa↓-YFP), and pcDNA3::α2Δα3δ-adrenergic receptor and processed for BRET measurements as described previously (34, 35). Cells were distributed into gray 96-well OptiPlates in triplicate (∼1 × 10⁵ cells/well). Luminescence was measured in the 480- and 530-nm emission windows at 2.5-s intervals beginning immediately after the addition of coelenterazine H to the sample wells. At 150 and 270 s, UK-14304 (1 μM) and rauwolscine (10 μM) were added by injection, respectively. Signal measurements were continued for 390 s following the addition of antagonist.

Cell Imaging—HEK-293 and COS-7 cells were seeded onto sterile 25-mm polylysine-D-coated coverslips 24 h following transfection. Cells were then processed for imaging 24 h later as described elsewhere (38). Primary antibodies were used at a dilution of 1:200 in cell washing solution (137 mM NaCl, 2.6 mM KCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4), and secondary
antibodies (goat anti-rabbit AlexaFluor488 or mouse AlexaFluor594, highly cross-adsorbed, Molecular Probes) were used at a dilution of 1:2000 in cell washing solution. All antibody dilutions were centrifuged at 10,000 × g for 10 min prior to use. The nucleus was stained with 1 μg/ml DAPI at the last washing step. Slides were then mounted with glass coverslips and analyzed with a Leica CTR5500 deconvolution fluorescence microscope using a 40× or 63× oil immersion objective as described elsewhere (38). All images were obtained from approximately the middle plane of the cells. Selected enlarged images were processed by deconvolution for higher resolution using Simple PCI version 6.6.0.0 (Hamamatsu Corp., Sewickley, PA). Images were evaluated for colocalization of GA marker proteins and AGS3 by three different approaches. First, each experiment was visually examined by at least two individuals to identify and count the population of cells exhibiting clear juxtanuclear enrichment of AGS3 and overlap of the AGS3 with GA marker proteins. Cells undergoing division were not counted to avoid any nonclarity in regard to data interpretation and the GA fragmentation that occurs during mitosis. 

FIGURE 2. Influence of cell surface receptor activation on the subcellular distribution of AGS3. HEK-293 (A) or COS-7 (B) cells expressing pEGFP::AGS3 (25 ng - HEK, 100 ng - COS-7), pcDNA3::Gαq11 (750 ng), and pcDNA3::α2A-ΔAR (750 ng) were incubated with vehicle or UK-14304 (10 μM) for 10 min and processed for fluorescent microscopy. The Golgi marker GM130 (red) was detected by immunofluorescence as described under “Experimental Procedures.” The images shown are representative of 10 (HEK) or three (COS-7) separate experiments. The arrowheads indicate juxtanuclear localization of AGS3 following receptor activation. Bar, 10 μm. Bottom panels, the relative subcellular distributions of AGS3-GFP and GM130 were quantified by fluorescence intensity scans (green, AGS3-GFP; red, GM130) through the cell, as indicated by the lines in the images and as described under “Experimental Procedures.”
cells expressing AGS3-GFP were examined in each experiment, and each experiment was repeated at least three times. Second, selected images were examined by scanning of fluorescence intensity across the cell using ImageJ (39). Third, images were also evaluated for colocalization using the Coloc_2 module within the Fiji image processing package (40), which provides a thresholded Pearson’s correlation coefficient for pixel overlap based on the approach developed by Costes et al. (41).

In cells transfected with pEGFP::ST, the relative distribution of ST-GFP, which resides in the trans-Golgi network, was determined by counting the numbers of ST-GFP puncta in the presence and absence of agonist. Similar experiments were conducted in cells expressing ST-GFP alone or together with pcDNA3::Gα13 and pcDNA3::α2A/1D-AR.

Data Analysis—Data were analyzed by analysis of variance, and significant differences between groups were determined by the Tukey a posteriori test using GraphPad Prism.

FIGURE 3. Influence of cell surface receptor activation on the subcellular distribution of AGS3 and the GA marker proteins TGN46 and mannosidase II. HEK-293 cells transfected with pEGFP::AGS3 (25 ng), pcDNA3::Gα13 (750 ng), and pcDNA3::α2A/1D-AR (750 ng) were incubated with vehicle or UK-14304 (10 μM) for 10 min and processed for fluorescent microscopy. The Golgi markers TGN46 (trans-Golgi network) (A) and Mann-II (luminal GA) (B) were detected by immunofluorescence as described under “Experimental Procedures.” The images shown are representative of 5–10 separate experiments. Bar, 10 μm. The relative subcellular distributions of AGS3-GFP and TGN46 and Mann-II were quantified by fluorescence intensity scans (green, AGS3-GFP; red, GM130) through the cell, as indicated by the lines in the images and as described under “Experimental Procedures.”
version 4.03 for Windows (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

We established a platform to monitor the interaction of GPR proteins and Goi in the intact cell by BRET. This platform involves an energy donor (AGS3-Rluc) and acceptor (Goi-YFP). Because AGS3-Rluc may bind up to four Goi-YFP, the BRET signal is particularly robust (34). At least three different types of GPR-Goi, complexes (AGS3-Goi, AGS4-Goi, and RGS14-Goi) are regulated by activation of a cell surface, G-protein-coupled receptor as well as by the non-receptor guanine nucleotide exchange factor Ric-8A and/or GIV (24, 33–36). Activation of the Goi-AR rapidly decreased AGS3-Rluc-Goi-YFP BRET to a new steady state, which was rapidly reversed by the addition of the Goi-AR antagonist rauwolscine (Fig. 1A). Based upon previous studies, it appears that the AGS3-Rluc is “released” from its membrane site, whereas Goi11-YFP remains tethered to the plasma membrane (34).

We hypothesized that the tethered AGS3 was released to play a role in signal propagation. As a first approach to test this hypothesis, we determined the subcellular location of AGS3 after its release from the cell cortex. Upon agonist treatment in HEK cells, the marked cortical distribution of AGS3 was lost in >90% of the cells (Fig. 1B), and many cells exhibited a robust juxtanuclear enrichment of AGS3 that appeared to overlap with the cis-Golgi apparatus-resident protein GM130 (Fig. 2A). Similar results were obtained in COS-7 cells (Fig. 2B). Similar results were also obtained in both HEK and COS7 cells expressing Goi11.4 The juxtanuclear localization of AGS3-GFP observed in the presence of agonist also overlapped with the trans-Golgi marker TGN46 and a marker of the luminal GA (mannosidase II) (Fig. 3). To further define the juxtanuclear localization of AGS3 observed upon the addition of agonist, we also scanned fluorescence intensities across cells for AGS3-GFP and GA marker proteins (Figs. 2 and 3). The fluorescence intensity scans indicated that AGS3 was generally enriched at the cell cortex in the absence of agonist. However, upon receptor activation, many cells exhibited a robust accumulation of AGS3 at the juxtanuclear region and spatial overlap with the GA marker proteins (Figs. 2 and 3).

The degree of colocalization of AGS3 with GA marker proteins following agonist treatment was quantitatively examined in AGS3-transfected cells using the Fiji image analysis suite. The robust, juxtanuclear localization of AGS3 after agonist treatment was visually obvious in many cells, whereas some cells exhibited a more diffuse intracellular distribution of AGS3, which was clearly different from the predominantly cortical distribution in the absence of agonist (Fig. 4A). Analysis of this mixed population of cells observed in the presence of agonist yielded a thresholded Pearson’s correlation coefficient for colocalization analysis with GM130 and AGS3-GFP of 0.58 ± 0.05 (n = 34) with agonist incubation versus 0.08 ± 0.04 (n =

4 S. S. Oner and S. M. Lanier, unpublished observations.
Further colocalization analysis of these two general subpopulations of cells with the GA marker GM130 indicated that the cells with robust, juxtanuclear localization of AGS3 following agonist treatment exhibited a thresholded Pearson’s correlation coefficient of 0.81 ± 0.02 (n = 16), whereas cells exhibiting a more diffuse distribution of AGS3 without any clear enrichment at the GA exhibited a thresholded Pearson’s correlation coefficient of 0.37 ± 0.04 (n = 18) (Fig. 4B). This analysis indicates that agonist treatment results in clear, statistically distinct, colocalization of AGS3 with the GA marker proteins. Similar results were obtained when colocalization analysis was conducted for AGS3 and the GA marker proteins TGN46 and mannosidase II (Fig. 4B).

The population of agonist-treated cells exhibiting the lower thresholded Pearson’s correlation coefficient (Fig. 4, A and B) probably reflects a graded response that may represent an intermediate step in translocation of AGS3 to the juxtanuclear region or represent other trafficking pathways for AGS3 (19, 23, 24, 38). For the purposes of this current study, we focused on the population of cells exhibiting the robust, juxtanuclear enrichment of AGS3 overlapping with GA marker proteins as defined by visual examination and/or thresholded Pearson’s correlation coefficients greater than 0.7, which represented ~50% of the cells expressing transfected AGS3 (Fig. 4, B and C). Analysis of enlarged fluorescent images with the three GA marker proteins and AGS3-GFP by deconvolution indicated that the translocated AGS3-GFP generally had strong spatial colocalization with TGN46, exhibiting a tubero-vesicular appearance perhaps related to protein secretion at the trans face of the GA (Figs. 2 and 3). Although there is clear overlap of AGS3-GFP with the GA marker proteins, there are distinct areas where the GA marker proteins GM130 and Mann-II do not overlap with AGS3, indicating that AGS3 may translocate to specific subcompartments within the GA (Figs. 2 and 3). Although there are many different organelles and functional compartments that position themselves at juxtanuclear sites, the clear spatial overlap with the three GA marker proteins is consistent with translocation of AGS3 to the GA following receptor activation. We also examined the potential distribution of AGS3 among early endosomes (EEA1), lysosomes

FIGURE 5. Comparative subcellular distribution for AGS3 and markers for early endosomes or lysosomes following receptor activation. HEK-293 cells expressing pEGFP::AGS3 (25 ng), pcDNA3::Gα13 (750 ng), and pcDNA3::α2AR (750 ng) were incubated with vehicle or UK-14304 (10 μM) for 10 min and processed for fluorescent microscopy as described under “Experimental Procedures.” The early endosome marker EEA1 (A) and lysosome marker LAMP2 (B) were detected by immunofluorescence as described under “Experimental Procedures.” The images shown are representative of three separate experiments. The relative subcellular distributions of AGS3-GFP and EEA1 and LAMP2 were quantified by fluorescence intensity scans (green, AGS3-GFP; red, EEA1 or LAMP2) through the cell as indicated by the lines in the images and as described under “Experimental Procedures.”
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FIGURE 6. Time course and mechanisms involved with the translocation of AGS3 to the Golgi apparatus. A, HEK-293 cells expressing pEGFP::AGS3 (25 ng), pcDNA3::G_{i3} (750 ng), and pcDNA3::α_{2A/D-AR} (750 ng) were incubated with UK-14304 (1 μM) for different time periods and processed for fluorescent microscopy. The images shown are representative of three separate experiments. The arrowheads indicate juxtanuclear localization of AGS3 following receptor activation. Red, GM130. Bottom, the relative subcellular distributions of AGS3-GFP and GM130 were quantified by fluorescence intensity scans (green, AGS3-GFP; red, GM130) through the cell, as indicated by the lines in the images and as described under “Experimental Procedures.” B, left, agonist-induced translocation of AGS3-GFP to the Golgi apparatus as determined by co-localization with GM130. Reversibility of the UK-14304-induced translocation of AGS3 was assessed by the addition of the antagonist rauwolscine (Rauw) (10 μM) following a 10-min incubation of the cells with UK-14304 (1 μM). Rauwolscine incubation was continued for 10 min, and then the cells were processed for fluorescent microscopy as described under “Experimental Procedures.” Another group of cells was treated with vehicle or 100 ng/ml pertussis toxin for 16 h prior to incubation with agonist. Data are expressed as means ± S.E. (error bars) (n = 3–7). Middle, influence of the phospholipase C inhibitor U73122 on agonist-induced translocation of AGS3 to the Golgi apparatus as determined by co-localization with GM130. HEK-293 cells expressing pEGFP::AGS3 (25 ng), pcDNA3::G_{i3} (750 ng), and pcDNA3::α_{2A/D-AR} (750 ng) were pretreated with U73122 (10 μM) or an inactive analog U73343 (10 μM) for 20 min prior to the addition of the α_{2A/D-AR} agonist UK-14304. Incubation with agonist was continued for 10 min before the cells were processed for image analysis. Data are expressed as means ± S.E. (n = 3). Right, quantitative analysis of cells exhibiting juxtanuclear AGS3-GFP co-localization with GM130. HEK-293 cells expressing pEGFP::AGS3 (25 ng), pcDNA3::G_{i3} (750 ng), and pcDNA3::α_{2A/D-AR} (750 ng) were incubated with vehicle or brefeldin A (BFA) (10 μg/ml) for 30 min, and agonist (UK-14304, 1 μM) was then added with incubation continued for 10 min. GM130 (red) was detected by immunofluorescence. Data are expressed as means ± S.E. (n = 3). *p < 0.0001 compared with control value in the absence of agonist. #, p < 0.0001 compared with value obtained in the presence of agonist. C, left, agonist-induced translocation of AGS3-GFP to the juxtanuclear area. HEK-293 cells expressing pEGFP::AGS3 (25 ng), pcDNA3::G_{i3} (750 ng), and pcDNA3::α_{2A/D-AR} (750 ng) were pretreated with vehicle or U0126 (20 μM) for 1 h. Cells were then treated with UK-14304 for 10 min and processed for image analysis. Right, subcellular distribution of an AGS3 construct in which 24 serine/threonine residues in the GPR domain were mutated to alanine (AGS3-PM). HEK-293 cells expressing pEGFP::AGS3-PM (25 ng) or pEGFP::AGS3-PM (25 ng) in the presence of pcDNA3::G_{i3} (750 ng) and pcDNA3::α_{2A/D-AR} (750 ng) were treated with the agonist UK-14304 for 10 min and processed for image analysis. Data are expressed as means ± S.E. (n = 3). PM, phosphomutant. In B and C, the numbers above the bars in parentheses indicate the number of AGS3-GFP-expressing cells examined.

(LAMP2), and the endoplasmic reticulum (calnexin). The subcellular distribution of these three organelle markers is much broader than the juxtanuclear localization of AGS3 observed following agonist treatment (Fig. 5).4

We initiated a series of experiments to understand the mechanism of AGS3 translocation and to further test our hypothesis. We first determined the time course for the translocation of AGS3 to the GA. AGS3-GFP translocation to the GA was observed within 30 s of the agonist addition and was maintained in GA for at least 30 min (Fig. 6A). The percentages of AGS3-GFP-expressing cells exhibiting agonist-induced translocation of AGS3 to the GA at different time points were 31.6 ± 2.7% (thresholded Pearson’s correlation coefficient = 0.88 ± 0.02, n = 4) (0.5 min), 39.4 ± 1.2% (thresholded Pearson’s correlation coefficient = 0.87 ± 0.03, n = 5) (1 min), 49 ± 2% (thresholded Pearson’s correlation coefficient = 0.84 ± 0.03, n = 4) (2 min), and 46.5 ± 2.8% (Pearson’s correlation coefficient = 0.81 ± 0.33, n = 6) (30 min). The spatial overlap of AGS3 with the GA marker protein GM130 was also observed by fluorescence intensity scanning across individual cells (Fig. 6A, bottom).

We then asked if agonist-induced translocation of AGS3 to the GA was reversible. In this series of experiments, cells were first incubated with the α_{2}-AR UK-14304 (1 μM) for 10 min to induce AGS3 release and translocation. At this point, the α_{2}-AR
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antagonist rauwolscine (10 μM) was added for 10 min with subsequent processing of the cells for visualization. As indicated in Fig. 6B, the GA translocation of AGS3 was reversed by the receptor antagonist. We also asked if pertussis toxin treatment, which ADP-ribosylates Goα, effectively uncoupling it from the receptor, altered the release and translocation of AGS3. The agonist-induced translocation of AGS3 to the GA was completely blocked by pertussis toxin treatment (Fig. 6B). These data are consistent with the reversibility of the effect of receptor activation on AGS3-Rluc-Gα1-i-YFP BRET (Fig. 1) and its sensitivity to pertussis toxin (34).

We then further investigated various signaling pathways to define the mechanisms of AGS3 translocation. The agonist-induced translocation of AGS3 to the GA was completely blocked by inhibition of phospholipase Cβ with U73122 (Fig. 6B). The effect of U73122 was specific, because its inactive analog U73343 had no effect (Fig. 6B). Furthermore, the agonist-induced translocation of AGS3 to the juxtacnnular region was not observed following treatment of cells with brefeldin A, which disrupts the structure of the GA (Fig. 6B). The precise mechanism by which phospholipase Cβ may regulate this transport event is not clear. Although U73122 is typically used as a phospholipase Cβ inhibitor, the molecule may have a number of other actions that contribute to its pharmacological profile (42). Nevertheless, U73122 should be a useful tool to dissect the mechanisms involved in AGS3 translocation going forward.

The subcellular distribution of AGS3 and its interaction with Go may be influenced by AGS3 phosphorylation (26, 63). However, a similar agonist–induced translocation of AGS3 to the GA was observed for an AGS3 construct in which 24 serine or threonine residues in the GPR domain were mutated to alanine to disrupt phosphorylation (Fig. 6C) (26). The agonist-induced translocation of AGS3 to the GA was also not altered by the MEK inhibitor U0126 (Fig. 6C).

To further dissect the mechanism of agonist-induced translocation of AGS3 to the GA, we examined the role of the AGS3 TPR and GPR domains. Both the TPR and GPR domains of AGS3 are important determinants of its subcellular distribution (34, 38, 43). Interaction of Goα with the GPR domains stabilizes AGS3 and other GPR proteins at the cell cortex, where it senses activation of a cell surface receptor (34–36). We first asked if GPR proteins lacking TPR domains (Fig. 7A) exhibited a similar agonist-induced translocation to the GA. The transcript variant AGS3-SHORT lacks the TPR domain but contains three GPR motifs (43). AGS4 is a group II AGS protein that has three GPR motifs and lacks a TPR domain (44). Although both AGS3-SH and AGS4 interact with Goα and are stabilized at the cell cortex, where they sense receptor activation (34, 35), neither protein was translocated to the GA upon receptor activation (Fig. 7A). Agonist-induced translocation of AGS3 to the GA was not observed for AGS3-Q/A-GFP (Figs. 7A), which cannot bind Goα and thus is not stabilized at the cell surface, where it can sense receptor activation (34, 38).

As noted above, neither AGS4 or AGS3-SHORT translocated to the GA upon receptor activation, suggesting that the TPR domain was required for accumulation of AGS3 at the GA upon the addition of agonist. We thus addressed the role of individual TPR motifs in GA translocation of AGS3 by determining the subcellular distribution of AGS3-GFP constructs lacking individual TPR motifs. AGS3 lacking TPR1 exhibited agonist-induced translocation similar to wild type AGS3. However, deletion of both TPR1 and TPR2 markedly reduced the agonist-induced accumulation of AGS3 in the GA (Fig. 7B). Similar results were observed with the progressive deletion of TPR3–7 (Fig. 7B). These data suggested that TPR2 or perhaps broader structural organization within the TPR domain was a key factor in the regulated translocation of AGS3. To further address this question, we generated AGS3-GFP constructs with single amino acid mutations of conserved residues within individual TPR motifs (38). Mutation of a single conserved G within individual TPR motifs (38).
for engagement with regulatory binding partners for AGS3 in the GA or in the context of the transport process.

We next asked if endogenous AGS3 also translocated to the GA. For endogenous AGS3, ~200 cells were examined in each of three experiments for localization of AGS3 and the GA marker protein GM130 with and without agonist treatment (Fig. 8). Upon receptor activation, endogenous AGS3 was clearly translocated to a juxtanuclear region, where it also localized with GM130 (Fig. 8). Each of the fluorescence intensity scans for Fig. 8 actually spans two cells, one of which exhibited AGS3 translocation to the GA and one of which did not. Of the 735 cells examined, 47.4 ± 2.4% exhibited GA localization of AGS3 following agonist treatment. The colocalization of endogenous AGS3 with GM130 was also examined by quantitative image analysis. The thresholded Pearson’s correlation coefficient for AGS3 and GM130 in responsive cells was 0.9 ± 0.02 (n = 10).

These data support the previous observations that the AGS3-Gαi signaling module at the cell surface senses receptor activation, and then the two binding partners dissociate (34). It is not known if the receptor directly couples to AGS3-Gαi or whether the coupling is indirect within the context of a larger signaling complex (see discussion in Ref. 34). Subsequent to release from the cell cortex, AGS3 is found in the GA. Receptor-mediated translocation of AGS3 is of great interest because it may have functional consequences with respect to protein secretion and trafficking through the GA, as suggested earlier by Groves et al. (18). Groves et al. reported that AGS3 overexpression redistributed TGN-localized proteins and modulated protein trafficking from the GA to the plasma membrane. siRNA-mediated knockdown of AGS3 also led to dispersal of TGN-localized proteins, including TGN46 and the mannose 6-phosphate receptor. These data suggest that AGS3 dynamically regulates events at the TGN.

We thus examined the functional consequences of agonist-induced translocation of endogenous AGS3 to the GA by monitoring the distribution of the specific trans-Golgi-resident protein sialyltransferase tagged with GFP (ST-GFP). In the absence of agonist, ST-GFP was tightly focused in the GA. However, agonist treatment (3-h incubation) disrupted the focused localization of ST-GFP with apparent vesiculation and fragmentation of the trans-Golgi network (Fig. 9, A and B). We also examined the distribution of AGS3 and the GA marker proteins GM130 and TGN46 following incubation of cells with agonist for 3 h. In general, the juxtanuclear localization of endogenous AGS3 or transfected AGS3-GFP was also observed following a 3-h agonist treatment (Fig. 10). However, in some instances, the juxtanuclear localization of AGS3 following longer incubation with agonist was more heterogeneous and not as compact as observed with shorter incubation times, consistent with an effect on overall GA architecture. In addition, the immunofluorescence intensity for TGN46 in the juxtanuclear region was consistently lower in cells exhibiting apparent GA accumulation of AGS3 following a 3-h agonist treatment (Fig. 10). This is most evident by comparing the intensity of juxtanuclear TGN46 with surrounding cells in Fig. 10A. The magnitude of the decrease in immunofluorescence intensity for

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TGN46 in cells exhibiting GA translocation of AGS3-GFP after a 3-h incubation with agonist varied as indicated in the different panels shown in Fig. 10A. The loss of TGN46, which is a marker of recycling endosomes, would suggest an effect of AGS3 on the cycling process and/or vesicle sorting at the TGN. In contrast to the observations with TGN46, the immunofluorescence intensity for GM130 was similar across all cells (Fig. 10, A and B).

The distribution of TGN46 within the cell is actually in apparent constant flux as it moves within the recycling endosome pathway. The kinetics of this flux are such that under standard conditions, the protein is accumulated at the trans-Golgi, providing a robust signal as a TGN marker. Upon disruption of the TGN or interference with the recycling endosome pathway, the accumulation of TGN46 at the trans-Golgi network is not observed because the protein is apparently dispersed through the cell, resulting in a loss of the robust immunofluorescence signal at the TGN. The reduction in the accumulation of TGN46 at the TGN following 3-h incubation with the α2-AR agonist UK-14304 probably reflects an effect on vesicle cycling events at the TGN rather than a reduction of TGN46 protein in the cell. (TGN46 dispersal also occurs with cell division, brefeldin A, and other interventions that disrupt the TGN. TGN46 immunoblots from vehicle- or agonist-treated cells indicated no marked change in the levels of TGN46 following agonist treatment, consistent with its apparent dispersal rather than actual loss of the protein).4

These data are consistent with the hypothesis that the AGS3•Goi complex acts as a signal transducer mediating downstream functional responses to activation of cell surface receptors. A primary downstream target of AGS3 is the trans-Golgi network, where it appears to modulate protein secretion and/or endosome recycling. The first suggestion of a direct effect of AGS3 on GA function was the report by Groves et al. (18), indicating that either a reduction or an increase in AGS3 expression resulted in altered GA structural organization. In addition, Groves et al. (18) reported regulation of the trafficking of a specific subgroup of proteins to the cell surface following siRNA-mediated reduction of AGS3. These data and the present work suggest that the receptor activation-induced dissociation of an AGS3•Goi complex with the subsequent docking of AGS3 at the GA provides an unexpected platform for regulation of events at the GA.

However, although altered AGS3 expression may influence GA structure and/or the movement of proteins through the secretory pathway, the observed effect of agonist on the TGN organization reported in the present paper may not be solely due to AGS3 translocation to the GA. The regulation of protein processing and movement in the secretory pathway is complex and involves multiple regulatory mechanisms and checkpoints. Specific subtypes of Gαi and Gβγ and various proteins that interact and regulate G-proteins are found in the GA, where they may influence basal and regulated processing of proteins through the secretory pathway (45–61). Several different factors may act in concert to influence membrane dynamics and vesicle recycling, sorting, and budding at the TGN.

Activation of M2 and M3 muscarinic receptors, which couple to heterotrimeric Gαi, and Gαq, respectively, leads to translocation of specific Gγ subtypes to the GA as Gβγ complexes (57). As reported for AGS3 in the present paper, the translocation of Gβγ to the GA leads to alterations in TGN organization and the processing of proteins through the secretory pathway (57). Protein kinase D and phospholipase Cβ, both of which are activated by Gβγ, play central roles in transport vesicle budding at the trans-Golgi network. Gβγ-mediated regulation of protein kinase D leads to GA fragmentation (49, 59–61). The Gβγ-mediated regulation of GA fragmentation may involve activation of phospholipase Cβ as it is blocked by U73122 (57, 60, 61). It is not clear if the phospholipase Cβ involved resides at the plasma membrane or within the GA, but the diacylglycerol generated upon its activation is postulated to be important for pro-

FIGURE 9. AGS3 translocation to the Golgi apparatus and distribution of the trans-Golgi protein ST-GFP. HEK-293 cells expressing pEGFP::ST (sialyltransferase) (ST-GFP) (50 ng) alone or together with pcDNA3:Goi3 (750 ng) and pcDNA3:α2aAR (750 ng) were incubated with agonist UK-14304 (10 μM) for 3 h and processed for fluorescent microscopy as described under “Experimental Procedures.” The images shown are representative of 3–5 separate experiments. B, quantitative analysis of percentage of the cells with the indicated vesicle numbers. Data are expressed as means ± S.E. (error bars) (n = 3), *, p < 0.0001 compared with control value in the absence of agonist. The numbers above the bars indicate the number of ST-GFP-expressing cells examined.
FIGURE 10. **AGS3 translocation to the Golgi apparatus following 3-h incubation with vehicle or the \( \alpha_2 \)-adrenergic receptor agonist UK-14304.**

**A**, HEK-293 cells expressing pEGFP::AGS3 (25 ng) together with pcDNA3::G\(_{\alpha}\)/H9251 (750 ng) and pcDNA3::D-AR (750 ng) were incubated with vehicle or agonist UK-14304 (10 \( \mu \)M) for 3 h and processed for fluorescence microscopy. The Golgi marker protein TGN46 was detected by immunofluorescence. Three different image fields are presented for cells incubated with agonist. **B**, HEK-293 cells expressing pcDNA3::G\(_{\alpha}\)/H9251 (750 ng) and pcDNA3::D-AR (750 ng) were incubated with vehicle or agonist UK-14304 (10 \( \mu \)M) for 3 h and processed for fluorescence microscopy. Endogenous AGS3 (red) and the Golgi marker protein GM130 (green) were detected by immunofluorescence as described under “Experimental Procedures.” Two different image fields are presented for cells incubated with agonist. The lower image panel was generated in parallel from HEK-293 cells transfected with pEGFP::AGS3 (25 ng) together with pcDNA3::G\(_{\alpha}\) (750 ng) and pcDNA3::D-AR (750 ng). The images shown in **A** and **B** are representative of three separate experiments.
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protein kinase D activation in the GA and the subsequent shutting of proteins out of the trans-Golgi network.

Thus, AGS3, Gαi, and Gβγ can all influence the structure and function of the GA. The role of AGS3 in these regulatory events is of particular interest. Via its GPR motifs, translocated AGS3 may regulate the interaction of Golgi-resident Gα with translocated Gβγ, increasing the availability of free Gβγ for effector engagement in the GA. Alternatively, the translocated AGS3 may interact with specific TPR-binding partners in the GA to engage in the GA. We also appreciate the continued technical assistance provided by Heather Bainbridge.

The regulated positioning of AGS3 in the GA adds an unexpected additional mechanism for dynamic and reversible regulation of protein trafficking through the secretory pathway. As noted above, AGS3 is implicated in various functional responses in several different tissues or cell types, suggesting that it is regulating a basic cellular process. Perhaps the regulation of GA dynamics and the secretory pathway is a target for AGS3 shared across multiple tissues.

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