Phosphatidylinositol 4 phosphate (PI4P) is highly enriched in the trans-Golgi network (TGN). Here we establish that PI4P is a central regulator of the recruitment of the GGA clathrin adaptor proteins to the TGN and that PI4P has a novel role in promoting their recognition of the ubiquitin (Ub) sorting signal. Knockdown of PI4KIIa by RNA interference (RNAi), which depletes the TGN’s PI4P, impaired the recruitment of the GGAs to the TGN. GGAs bind PI4P primarily through their GAT domain, in a region called C-GAT, which also binds Ub but not Arf1. We identified two basic residues in the GAT domain that are essential for PI4P binding in vitro and for the recruitment of GGAs to the TGN in vivo. Unlike wild-type GGA, GGA with mutated GATs failed to rescue the abnormal TGN phenotype of the GGA RNAi-depleted cells. These residues partially overlap with those that bind Ub, and PI4P increased the affinity of the GAT domain for Ub. Because the recruitment of clathrin adaptors and their cargoes to the TGN is mediated through a web of low-affinity interactions, our results show that the dual roles of PI4P can promote specific GGA targeting and cargo recognition at the TGN.

INTRODUCTION

Phosphatidylinositol 4 phosphate (PI4P) has recently emerged as a trans-Golgi network (TGN) marker that establishes a signpost for the recruitment of trafficking proteins (Behnia and Munro, 2005; Carlton and Cullen, 2005). Recently, we found that the major TGN clathrin adaptor, AP-1, binds PI4P and requires PI4P for TGN targeting (Wang et al., 2003; Heldwein et al., 2004). PI4P is particularly enriched at the TGN (Wang et al., 2003), and additional TGN-localized proteins, such as epsinR (Hirst et al., 2003), OSBP (Levine and Munro, 2002), and FAPP (Godi et al., 2004), that bind PI4P have also been identified. Because these TGN-enriched adaptor proteins bind both PI4P and the small GTPase ADP-ribosylation factor binding protein 1 (Arf1), we and others proposed that PI4P and Arf1 are essential components of a coincidence detection network that specifies their recruitment to the TGN (Wang et al., 2003; Godi et al., 2004; Behnia and Munro, 2005; Carlton and Cullen, 2005).

This paradigm begs the question of whether the Golgi-localized, γ-ear containing, Arf-binding proteins (GGAs), a major family of TGN-enriched adaptors that mediate trafficking between the TGN and endosomes (Bonifacino, 2004), also use PI4P to ensure organelle-specific targeting. It has been clearly established that GGA association with the TGN is dependent on the small GTPase Arf1 (Puertollano et al., 2001; Takatsu et al., 2002; Collins et al., 2003) and that the Arf1 binding site is located at the NH2-terminus of the GAT (GGA and Tom1) domain (Shiba et al., 2003; Bildeau et al., 2004; Kawasaki et al., 2005; Prag et al., 2005). This domain is sandwiched between the VHS (Vps27, Hrs, STAM) and the appendage domains in GGA’s linear sequence (see Figure 2A). The VHS domain binds to the acidic cluster-dileucine motifs found on cargo proteins (Doray et al., 2002; Puertollano et al., 2001; see Figure 2A).

Humans have three GGAs (GGA1, GGA2, and GGA3). GGA1 and 3 bind monoubiquitin (Ub) and may therefore sort ubiquitinated cargoes (Bildeau et al., 2004; Puertollano and Bonifacino, 2004; Scott et al., 2004). Saccharomyces cerevisiae has two Gga proteins, called Gga1p and 2p, which also sort ubiquitinated cargo proteins between the TGN and endosomes (Bonifacino, 2004; Scott et al., 2004; Kim et al., 2007). GGAs have two Ub-binding sites that are located downstream of the Arf1-binding site in the GAT domain (Bildeau et al., 2004; Mattera et al., 2004; Puertollano and Bonifacino, 2004; Shiba et al., 2004; Kawasaki et al., 2005; Prag et al., 2005; see Figure 2, A and B).

In this article, we examine the role of PI4P in the recruitment of the GGAs. We establish that PI4P is essential for...
their TGN recruitment and that PI4P also promotes GGA binding to ubiquitinated cargoes.

MATERIALS AND METHODS

Antibodies and Reagents

Affinity-purified rabbit polyclonal anti-PI4KIIa was generated as described previously (Wei et al., 2002). Other antibodies were obtained as follows. Polyclonal antibodies: sheep anti-TGN46 (Serotec, Raleigh, NC), goat anti-GST (Amersham Pharmacia Biotech), rabbit anti-V5 (Invitrogen), anti-His (BD Transduction Laboratories, Lexington, KY), anti-actin (Sigma, St. Louis, MO). Most of the other reagents were from Sigma, except as noted in the text.

Cell Culture and Plasmid Transfections

HeLa cells were cultured in DMEM with 10% (vol/vol) fetal bovine serum (FBS), 10 mM HEPES, and 1 mM sodium pyruvate at 37°C. They were transiently transfected with plasmid and lipofectAMINE 2000 (Invitrogen, Carlsbad, CA), and cells were processed for immunofluorescence 18–24 h later.

RNA Interference

The PI4KIIa small interfering RNA (siRNA) primers used were as described previously (Wang et al., 2003). HeLa cells were plated in six-well plates at between 30 and 40% confluence for 24 h and transfected with 10 μl of 20 μM siRNA and 3 μl Oligofectamine (Invitrogen) in 1 ml of Opti-MEM. After 5 h, cells were rinsed and cultured in DMEM containing 10% FBS. Cells were fixed with 4% paraformaldehyde and permeabilization in 0.1% Triton X-100 on ice for 5 min. In the case of Arf, staining, cells were fixed with methanol at −20°C for 10 min. Fixed and permeabilized cells were incubated with antibodies in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 3% donkey serum. Immunofluorescence was detected by a Zeiss LSM510 laser scanning confocal microscope (Thornwood, NY) using a 63× oil 1.3 NA PlanApo objective. The GGA fluorescence intensity ratio of TGN/entire cell was obtained from cells in randomly chosen fields, using the Metamorph software (Universal Imaging, West Chester, PA), as described previously (Wang et al., 2003). The effect of GGA1 RNAi on the TGN morphology was examined by quantification or BamHI-SmaI sites of pCMV5/myc1 for overexpression in HeLa cells. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene, La Jolla, CA). PCRs were performed using an overlap extension technique and prepared by spotting serially diluted phosphoinositides (100, 50, and 25 pmol, from Echelon Biosciences) on Hybond-C extra nitrocellulose membrane (Amersham). Membranes were washed extensively with TBS containing 0.1% (vol/vol) Tween 20 before probing sequentially with primary antibodies and horseradish peroxidase–conjugated secondary antibodies. Bound immunocomplexes were identified with ECL Plus (Amersham) and quantitated using the Storm phosphorimager (GE Healthcare, Waukesha, WI).

Ub-Agarose Binding Assay

GGA GST-GAT or Gga1p His-VHS-GAT, respectively. GST-GAT was placed in the sample cell of a Microcal VP-ITC instrument at 50°C. The reaction was stopped by adding 2 mM MgCl2. Purified recombinant His-monoubiquitin (His-ub) was a gift of D. DeMartino (UT Southwestern) and bovine Ub was purchased from Sigma. The yeast Gga1p His-VHS-GAT was expressed in Rosetta cells (Novagen, Madison, WI) using standard protocols with IPTG induction at 30°C. Recombinant proteins were affinity purified on nickel nitrilotriacetic acid His-Bind Resin (Novagen) in Tris-buffered saline (TBS; 10 mM Tris, 140 mM NaCl, pH 8.0) with 5 mM imidazole and eluted with TBS with 150 mM imidazole.

Liposome Pulldown Assay

All lipids were from Sigma, unless otherwise specified. Mixed lipid vesicles containing 57% dioleoyl phosphatidylcholine (PC), 28% dipalmitoyl phosphatidyl ethanolamine (PE), and 15% of either phosphatidylinerine (PS), PI4P (Avanti Polar Lipids, Alabaster, AL), PI3P (Echelon Biosciences, Salt Lake City, UT), or PI(4,5)P2 (Echelon Biosciences) were prepared (Heldwein et al., 2003). They were dissolved in chloroform and resuspended in the appropriate ratio. Chloroform was evaporated under a slow stream of nitrogen, and the dried lipids were resuspended in cyclohexane and immediately placed in dry ice. The frozen mixture was dried in a lyophilizer, and the resulting dry powder was resuspended in TBS (10 mM Tris/Cl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol [DTT]) to 5 mg/ml by vortexing vigorously at room temperature to obtain a milky suspension. In most studies, 0.02 mg/ml recombinant GGA fragments were incubated with 0.2 mg/ml lipid vesicles for 15 min at final temperature in 50 μl of TBS. Liposomes were collected by centrifugation at 200,000 × g for 10 min, and after carefully removing the supernatant, the pelleted protein was dissolved in gel sample buffer. GGA domain proteins in the supernatants and pellets were stained with Coomassie blue after SDS-PAGE and quantitated by densitometry.

Protein-Lipid Overlay Assay

Recombinant GST-tagged GGA domains. His-tagged yeast GGA domain or His-tagged Ub was incubated with PIP-Arrays (Echelon Biosciences) or homemade lipid strips at 4°C overnight as described previously (Wang et al., 2003). "Homemade" lipid strips were prepared by boiling serially diluted phosphoinositides (100, 50, and 25 pmol, from Echelon Biosciences) on Hybond-C extra nitrocellulose membrane (Amersham). Membranes were washed extensively with TBS containing 0.1% (vol/vol) Tween 20 before probing sequentially with primary antibodies and horseradish peroxidase–conjugated secondary antibodies. Bound immunocomplexes were identified with ECL Plus (Amersham) and quantitated using the Storm phosphorimager (GE Healthcare, Waukesha, WI).

In Vivo Ubiquitination

Hela cells were cotransfected with myc-GGA1 and hemagglutinin (HA)-Ub cDNA (a gift from K. Orth, UT Southwestern) as described (Shiba et al., 2004). Myc-GGA1 was immunoprecipitated with anti-myc antibody, and ubiquitinated GGA1 was detected by Western blotting with anti-HA. The extent of ubiquitination of the immunoprecipitated GGA1 was quantitated as a ratio of the intensity of the HA-Ub and myc-GGA1 in the Western blot.

Isotothermal Titration Calorimetry

GGA GST-GAT and bovine Ub were dialyzed against PBS supplemented with 2 mM β-mercaptoethanol. Protein concentrations were determined using the extinction coefficients of 50, 900, and 1280 M−1 cm−1 for GST-GAT and Ub, respectively. GST-GAT was placed in the sample cell of a MicroCal VP isothermal titration calorimetry (ITC) instrument at 50 μM and Ub (0.5 μM

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initial concentration) was injected serially into the reaction chamber at 30°C. When appropriate, 50 μM water-soluble PI4P (diC8PPI4P, Echelon Biosciences) was incubated with GST-GAT1 and His-Ub separately at 30°C, before injection. Data were fitted by using the ORIGIN software (Origin-Lab, Northampton, MA).

RESULTS

**GGAs’ TGN Targeting Is PI4P Dependent**

To determine if PI4P is important for the recruitment of the GGAs to the TGN, we used RNAi to knockdown phosphatidylinositol 4 phosphate kinase IIα (PI4KIIα), a resident TGN protein that generates the bulk of the TGN’s PI4P (Barylko et al., 2001; Wang et al., 2003). In the absence of RNAi treatment, all three mammalian GGAs were concentrated at the TGN, which was identified by staining with anti-TGN46 (Figure 1, A–C, left panels). PI4KIIα depletion decreased the association of all three GGAs with the TGN (Figure 1, A–C, right panels), and induced redistribution of GGAs to punctuate and/or tubular endomembranes, as well as to the cytoplasm. GGA1 had the most particulate extra-Golgi staining, suggesting that it might associate with membranes somewhat better than GGA2 and 3 even in the absence of PI4P. The immunofluorescence images were used to estimate the extent of TGN association. The fluorescence intensity of the GGAs per unit area in the TGN was expressed relative to that for the entire cell to obtain a TGN “intensity ratio.” The intensity ratios for GGA1 in control and PI4KIIα RNAi cells are 9.57 ± 0.48 (n = 10) and 2.67 ± 0.16 (n = 10), respectively. Thus, there was a 72% decrease in the association of GGA1 with the TGN in the cells treated with PI4KIIα siRNA.

We also used biochemical fractionation to determine if PI4KIIα depletion altered the association of GGA1 with membranes. PI4KIIα RNAi decreased GGA1 association with the membrane pellet by 40 ± 7% (n = 3) and increased the amount recovered in the supernatant (Figure 1A, Western blot). In contrast, the partitioning of β-COP, a Golgi-enriched protein that does not bind PI4P, was not changed at all. Therefore, PI4KIIα depletion blocked GGA1 recruitment specifically. The 40% decrease in membrane association estimated by fractionation is less than the 72% decrease in TGN association estimated by immunofluorescence staining and is consistent with the fluorescence images showing that some GGA1 redistributed to extra-Golgi membranes after PI4KIIα depletion.

GGAs require Arf1-GTP for TGN localization (Puertollano et al., 2001; Ťakatsu et al., 2002; Collins et al., 2003). Therefore, we investigated the possibility that GGAs mislocalization in PI4KIIα RNAi-treated cells is secondary to a disruption of Arf1 activation. PI4KIIα RNAi had little effect on Arf1 localization in the perinuclear region of the cells (Figure 1D), as the intensity ratios for Arf in control and PI4KIIα RNAi cells are 3.00 ± 0.10 (n = 10) and 2.84 ± 0.14 (n = 9), respectively. Because only activated Arf1 binds the Golgi and TGN, PI4KIIα RNAi disrupts GGAs’ TGN targeting primarily by depleting PI4P and not by severely compromising Arf1 activation.

**GGA Binds PI4P Primarily through Its GAT Domain**

To identify the mechanism by which PI4P recruits GGAs to the TGN, we first determined if the GGA domains bind PI4P directly. Recombinant GST-GGA2 VHS-GAT, GAT, and VHS were incubated with mixed lipid vesicles containing either PI4P or PS. All three constructs bound PI4P, but GAT and 50% VHS sedimented. These results suggest that the GAT domain bound PI4P, whereas PI4P-GAT and 50% VHS-GAT sediments. These results suggest that the GAT domain bound PI4P, whereas PI4P-GAT and 50% VHS-GAT. Previous studies have shown that although VHS and GAT can function as independent modules, they can alter the binding of each other to select ligands when expressed in tandem (Hirsch et al., 2003; Mattera et al., 2004).

Binding was not simply due to charge–charge interactions, because only 10% of GAT sedimented with liposomes containing another basic lipid, PS (Figure 2C, SDS gel). The ratio of binding to PI4P versus PS liposomes from several experiments was calculated and is referred to as PI4P binding index in the bar graph shown in Figure 2C. GGA2 GAT binds PI4P liposome 6.8 ± 0.5 (mean ± SE, n = 5) times better than PS liposome, whereas VHS-GAT and VHS bound 3.3 ± 0.4 (n = 5) and 2.9 ± 0.5 (n = 6), respectively. Like GGA2 GAT, GGA1 GAT also bound PI4P mixed lipid vesicles in a dose-dependent manner, and there was no obvious difference between them (Figure 2D).

The preference of GAT for PI4P was confirmed by lipid dot blot assays. GGA2 GAT bound PI4P preferentially compared with other mono-, di-, or triphosphorylated phosphoinositides (Figure 2D). In agreement with the liposome pull-down assays, VHS did not bind PI4P as well as GAT. We
Figure 2. GGA GAT binding to PI4P. (A) GGA domain organization and structure. Left, the modular organization of the GGA domains. The GGA1 domain boundaries are as defined by Suer et al. (2003). The GAT domain is further divided into the N-GAT and C-GAT domains, which bind Arf1 and Ub, respectively. Our data showed that C-GAT also binds PI4P. Middle, a ribbon diagram of the X-ray crystal structure of GGA1 GAT, adapted from Collins et al. (2003). The GAT domain has four helices (named α1–4). The N-GAT domain has a hook-helix (α1 and part of α2), and the C-GAT domain has a three-helix bundle that is comprised of a portion of α2 and the entire α3 and α4. The PI4P-binding residues, GGA1 R260 and R265 (equivalent to GGA2 R276 and R281) are highlighted in red. We showed the GGA1 GAT structure instead of the GGA2 GAT structure here because only the former has been solved at the atomic level. Right, an expanded view of the GGA1 C-GAT structure. The C-GAT domain is rotated slightly in order to display the relation between the Ub-binding sites and PI4P-binding residues. Red, PI4P-binding residues; blue and green, Ub-binding residues in sites 1 and 2, as defined in yeast (Bilodeau et al., 2004). Additional site 1 residues identified by X-ray crystallography of the mammalian GGA GAT:Ub (Bilodeau et al., 2004; Kawasaki et al., 2005; Prag et al., 2005) that fall outside of these regions are not highlighted to simplify the presentation (see panel B). The site 1 residues were observed to bind Ub in the GGA3 GAT:Ub structure by Prag et al. (2005) and Kawasaki et al. (2005). The site 2 binding residues were identified...
therefore focused on identifying the minimal PI4P-binding domain in GAT.

**Identification of GAT’s Minimal PI4P-binding Domain**

The GAT domain has multiple binding partners. It binds Arf1-GTP through the NH2-terminal “hook” region (referred to as N-GAT; Collins et al., 2003; Suer et al., 2003; see Figure 2, A and B, for definition). It also binds Ub and Rabaptin 5 at its COOH-terminal triple helix bundle (C-GAT), which encompasses the C-terminal half of helix α2 and helices α3 and α4 (Bilodeau et al., 2004; Mattera et al., 2004; Kawasaki et al., 2005; Prag et al., 2005). The C-GAT domain bound PI4P in lipid dot blots (Figure 3A), whereas the N-GAT domain did not (data not shown). The GAT α3 + α4 helices together bound PI4P much better than either α3 or α4 individually (Figure 3A). Therefore, it is likely that the GGA3 bind PI4P through their GAT α3 and α4 helices.

**Identification of the GAT PI4P-binding Site**

Site-directed mutagenesis was used to identify the residues in these helices that are essential for PI4P binding. Phosphoinositides bind proteins through multiple interactions, including contacts between their negatively charged phosphate head groups and the side chains of basic amino acid residues (Yin and Janmey, 2003). GGA GAT α3 and α4 do not have obvious sequence or structural similarity to any of the known PI4P-binding motifs (Mao et al., 2001; Levine and Munro, 2002; Collins et al., 2003; Hirst et al., 2003; Godi et al., 2004; Heldwein et al., 2004; Yu et al., 2004). However, they have three highly conserved solvent exposed basic residues (GGA2 R276, R281, and Y310) that can potentially interact with PI4P’s phosphate groups (Figure 2, A and B; equivalent to GGA1 R260, R265, and Y294). These residues were mutated individually to glutamic acid (E) or alanine (A) for in vitro or in vivo studies.

In liposome pulldown assays, 89% of wild-type (wt) GAT sedimented with PI4P liposomes, whereas much less sedimented with liposomes containing other acidic lipids (Figure 3B). In contrast considerably less GAT R276E, R281E, and Y310E cosedimented with PI4P liposomes, and there was no significant difference in the amount bound to other acidic lipids (Figure 3B). These results suggest that these three basic residues may be essential for PI4P binding in vitro.

Although GGA2 R276, R281, and Y310 are far removed from the N-GAT region that binds Arf1 (Figure 2A), it is nonetheless important to evaluate the effect of their mutation on Arf1 binding. We adopted a PS liposome pulldown method that was used previously to show that Arf1-GTP promotes binding of the clathrin adaptor AP-1 complex to lipid vesicles (Heldwein et al., 2004). GGA2 GAT bound PS containing vesicles, and binding was increased by 2.2-fold by Arf1-GTP. Arf1-GTP also increased GGA2 GAT R276E and R281E binding to PS vesicles by a statistically significant extent (Figure 3C). Therefore, these mutations selectively impaired PI4P binding without affecting the ability of the GAT domain to interact with Arf1-GTP. Therefore, GGA2 R276 and R281 are important for PI4P binding in vitro. Both residues are conserved in the human GGAs and yeast Gga proteins (Figure 2B), which also bind PI4P (see Figure 5C).

In contrast, the GGA2 GAT Y310 mutation decreased PI4P binding (Figure 3B) and compromised the ability of Arf1 to promote GAT binding to liposomes (Figure 3C). These results suggest that the Y310E mutation may have changed the conformation of GAT, as would be consistent with the previous suggestion that Y310 contributes to the packing of the α2 + α3 + α4 three helix bundle (Shiba et al., 2004). In view of this change, we cannot conclude based on the current data that Y310 contributes to PI4P interaction.

**PI4P-binding Site Mutants Have Decreased Association with the TGN**

The GGA2 GAT PI4P binding mutants were expressed in cells to assess the impact of decreased PI4P binding on GAT recruitment to the TGN. As shown previously, the wt GAT domain was targeted to the TGN (Puertollano et al., 2001; Takatsu et al., 2002; Collins et al., 2003; Figure 4A). In contrast, GGA2 GAT R276E, R281E, and R281A were not TGN associated, even when expressed at low levels (e.g., see arrows in the R281A panel of Figure 4A). The expressed mutants were predominantly cytosolic, although some were also present in the nucleus. The intensity ratio in the TGN relative to the entire cell was 1.54 ± 0.07 (n = 10) for GGA2 GAT R276E compared with 4.79 ± 0.38 (n = 10) for wt GAT. GAT K311E, which bound PI4P normally in vitro (data not shown), was still enriched in the TGN (Figure 4A), with an intensity ratio of 3.64 ± 0.2 (n = 11). The strong correlation between the ability of the GAT domain to bind PI4P in vitro and to associate with the TGN in vivo establishes that the recruitment of the GAT domain to the TGN requires PI4P.

We also examined the behavior of the PI4P binding mutations in the context of the full-length GGAs. GGA2 R276E and R281A were mostly cytosolic (Figure 4B), confirming that these residues are important for TGN targeting of the full-length protein. Unlike the equivalent GATs (Figure 4A), they were not nuclear localized, and they were slightly more TGN associated. Overall, these results demonstrate that PI4P binding is essential for the TGN localization of the GGAs and that the other interactions mediated by the modules outside of the GAT domain are not sufficient to recruit the GGAs to the TGN in the absence of PI4P binding.

The decrease in mutant GGAs association with membrane was confirmed biochemically. High-speed centrifugation pelleted 10.4% of wt GGA2, but only 1.0 and 2.4% of GGA2 R276A and R281A, respectively (Figure 4B, right panel).
The GGA PI4P-binding Mutant Is Functionally Defective in Cells

To address the question of whether these PI4P binding mutations rendered the GGAs functionally defective, we used a GGA1 RNAi rescue protocol as a readout (Ghosh et al., 2003). After GGA1 was depleted by RNAi, the cDNA for wt and mutant GGAs were introduced to determine if they can rescue the knockdown phenotype. Normally, HeLa cells had a compact TGN, and only 12% of the control (mock) siRNA-transfected cells had an expanded TGN phenotype (Figure 4C). As shown previously (Ghosh et al., 2003), GGA1 depletion increased the percentage of cells with abnormally expanded TGN to 50%. This abnormal phenotype was rescued by overexpressing GGA1, as evidenced by the drop in the percentage of cells with expanded TGN back to 8% (Figure 4C). As shown previously (Ghosh et al., 2003), GGA1 depletion increased the percentage of cells with abnormally expanded TGN to 50%. This abnormal phenotype was rescued by overexpressing GGA1, as evidenced by the drop in the percentage of cells with expanded TGN back to 8% (Figure 4C). GGA1 R260A (which is equivalent to the GGA2 R276A) was not targeted to the TGN, and, significantly, it was not able to rescue the expanded TGN phenotype (42% abnormal TGN) of GGA1-depleted cells (Figure 4C). Similar results were obtained with the GGA1 R265A mutant (equivalent to GGA2 R281A, data not shown). Therefore, these PI4P binding mutants are functionally defective in the in vivo context.

GGA's TGN Targeting Has a Dual Requirement for PI4P and Arf1

Because PI4P binding is necessary for GGA's TGN recruitment and function in cells, we asked if PI4P binding per se is sufficient for the GGAs' TGN localization. We also reexamine the notion that Arf1 binding is sufficient for GAT targeting (Collins et al., 2003). This model was based on an in vitro recruitment assay in which N-GAT, which binds Arf1 but not PI4P, was added to permeabilized cells in the presence of cytosol and GTPH9253S. Neither GGA2 C-GAT, which binds PI4P but not Arf1, nor GGA2 N-GAT domain, which binds Arf1 but not PI4P, was concentrated in the perinuclear region (Figure 4D). The fluorescence intensity ratios of TGN/entire cell for GAT, C-GAT, and N-GAT were 4.8/110.0 (n = 10), 2.1 ± 0.1 (n = 13), and 1.8 ± 0.1 (n = 8), respectively. The 56 and 62% decrease in TGN association by C-GAT and N-GAT, respectively, showed that neither PI4P binding nor Arf1 binding alone was sufficient for TGN recruitment. Because TGN recruitment requires both N- and C-GAT, we conclude that the is a dual requirement for both Arf1 and PI4P interaction for the proper localization of GGAs at the TGN.
**PI4P Promotes GAT Binding to Ub**

We examined the effect of PI4P on the binding of the GAT domain to Ub. The rationale for these studies is as follows: first, PI4P and Ub both bind to the C-GAT domain (Kawasaki et al., 2005; Prag et al., 2005; Figure 2, A and B), but the extent of their overlap is unknown, and it is not possible to predict a priori whether the overlap would inhibit or promote these ligands’ interaction with the GAT domain; second, one of the PI4P-binding residues (GGA1 R260/GGA2 R276) may also be involved in binding to Ub, because its mutation decreased GAT binding to the Ub-agarose (Bilodeau et al., 2004; Mattera et al., 2004; Puertollano and Bonifacino, 2004; Shiba et al., 2004; Kawasaki et al., 2005; Prag et al., 2005). Third, GGA2 GAT binds Ub-agarose more weakly than the other GGAs (Puertollano and Bonifacino, 2004; Shiba et al., 2004), even though its amino acid sequence in the Ub-binding regions is highly homologous to that of the GAT domains of the other GGAs (Figure 2B). This difference raised the possibility that there may be isoform-specific preference for ubiquitin versus nonubiquitin sorting signals.

PI4P micelles promoted GGA1 binding to Ub-agarose in a dose-dependent manner (Figure 5A, left panel). PI4P, 20 μM, increased GGA1 GAT binding to Ub-agarose by 3.7 ± 0.8-fold (n = 3), and this increase is statistically significant (Figure 5B, left and middle). In contrast, PI3P, which has the same charge as PI4P, did not produce a statistically significant change (Figure 5B, left and middle). PI4P-containing mixed lipid vesicles also promoted Ub binding, whereas PS-containing liposomes did not (Figure 5B, right). GGA2
GAT, which bound Ub-agarose more weakly than GGA1 GAT, was also stimulated to bind Ub by PI4P (Figure 5A, middle). PI4P, which binds yeast Gga1p His-VHS-GAT directly on lipid dot blots (Figure 5C), also stimulated its binding to Ub-agarose (Figure 5A, right). PI4P increased GGA1 GAT binding to Ub by 3.73 ± 0.8-fold and PI3P 1.5 ± 0.3-fold (n = 3). *p = 0.024, NS, p = 0.775. (C) His-Ub did not bind PI4P on lipid dot blots. His-Ub (at 8 or 40 nM) and His-VHS-GAT from yeast Gga1p (at 8 nM) were incubated with lipid dots made in the laboratory (lipids: 100, 50, and 25 pmol), and bound proteins were detected with anti-His antibody. (D) PI4P did not promote mutant GAT binding to Ub-agarose. GGA1 GAT wt, R265A, and R260A (0.25 μM) were incubated with Ub-agarose in the presence of 15 μM PI4P. The proteins used for the binding studies are shown (input). (E) GGA1 ubiquitination in vivo. HeLa cells were cotransfected with myc-tagged wt or mutant GGA1 and HA-Ub cDNAs. Myc-GGA1 was immunoprecipitated and blotted with anti-myc antibody to detect GGA1 and anti-HA to detect Ub associated with GGA1. The extent of ubiquitination for each sample was expressed as a ratio of the intensity of the HA-Ub to myc-GGA1 signals in the myc-GGA1 immunoprecipitates, and the value for the wt GGA1 is defined as 1.0. Data shown are mean ± SE (n = 3). (F) ITC analysis. GGA1 GST-GAT and bovine Ub were each preincubated with 50 μM water-soluble diC8PI4P before mixing. The kcal/mol of Ub as a function of the molar ratio of Ub to GGA1 GAT is shown, and the curves were fitted by using a one-site model. The K_d values shown are mean ± SE of five experiments using several different preparations of GGA1 GAT. The insets show the heat change elicited by successive injections of Ub into a chamber containing the GAT1 solution.

The effect of PI4P on the interaction between the GAT domain and Ub was examined more quantitatively under true in-solution conditions by ITC. GGA1 GAT binding to Ub released heat (Figure 5F), and the binding isotherm fitted a one-site model that had an estimated K_d value of 26 ± 3 μM (n = 5; Figure 5F). Significantly, in the presence of diC8PI4P, the K_d value for GGA1 GAT:Ub binding decreased by 2.6-fold.

DISCUSSION

The findings presented here establish that GGA1 and GGA2 GAT both bind PI4P in vitro, and the association of all three mammalian GGAs with the TGN is PI4P dependent in vivo. PI4P and Arf1 are both necessary, and importantly, neither alone is sufficient for TGN targeting. Together with other's
findings on epsinR (Hirst et al., 2003), OSBP (Levine and Munro, 2002), and FAPP (Godi et al., 2004), we propose that PI4P is a major lipid platform for the recruitment of almost all of the currently known TGN-enriched clathrin adaptors. Although these adaptors have low estimated affinity for PI4P, their dual requirement for PI4P and Arf1-GTP allows them to act as sensitive coincidence detectors that associate with the TGN in a spatially and temporally appropriate manner (Carlton and Cullen, 2005).

GGAs bind PI4P primarily through their GAT domain. A comparison of the binding data suggests that the VHS domain may also bind PI4P, but to a lesser extent, and that VHS decreases GAT binding to PI4P in VHS-GAT. This finding is unexpected because it has been shown previously that GGA1 VHS-GAT has four times higher affinity for Arf1 than GAT1 alone (Hirsch et al., 2003). Our data here would suggest that VHS regulates Arf1 and PI4P binding in opposite directions. The significance of this difference in the context of the full-length GGA protein remains to be determined. Irregardless of whether PI4P binding by GAT is regulated by VHS, our result clearly demonstrates that GAT is important for GGA recruitment to the TGN. Mutations in the GAT domain show that two conserved basic residues in C-GAT are critical for PI4P binding in vitro (Figure 3B) for association of the full-length GGA with the TGN (Figure 4B) and for maintenance of TGN functions in vivo (Figure 4C). These residues are located in α3 of GAT’s three helix bundle (Figure 2, A and B). Mutation of either residue individually diminishes PI4P binding in vitro and TGN recruitment and function in vivo.

The structures of GGA1 GAT alone (Collins et al., 2003) show that GGA1 R260 and R265 are located on the solvent exposed face of the GAT three helix bundle (Figure 2A) and are therefore accessible to the charged phosphate headgroups on PI4P. However, they are separated by 13.7 Å, which is at the upper limit of the span that can be reached by a single PI4P molecule; PI4P is estimated to be ~8 Å in its longest dimension, and it together with two salt bridges can potentially span 14 Å. Moreover, these residues project in different directions from the GAT helix bundle (Figure 2A). Therefore, the most straightforward interpretation is that these two residues bind different PI4P and that binding to multiple PI4P increases the binding avidity. On the other hand, if they were to bind the same PI4P, at least one residue will have to move into sterically favorable interaction distance. Allosteric regulation of GGA by phosphorylation has been reported previously (Ghosh and Kornfeld, 2003; McKay and Kahn, 2004), and a PI4P-induced conformational change is not precluded by the current structures of the GAT domain.

Irrespective of how GGA binds PI4P, it is remarkable that despite GGA’s engagement in the extensive network of parallel interactions at the TGN, a single point mutation in GGA’s PI4P headgroup interactive site is sufficient to almost completely abrogate correct intracellular targeting. PI4P binding is therefore a critical upstream component of the GGA recruitment cascade.

GGAs bind Ub, which is found on an increasing number of cargo proteins that traffic in the anterograde and retrograde directions (Dell’Angelica et al., 2000, 2002; Wahle et al., 2004). PI4P promotes the recognition of the Ub-sorting signal by mammalian and yeast GGAs (Figure 5). Binding of one ligand (PI4P) may confer entropic advantage to the binding of another ligand (Ub). This scenario is different from that proposed for Ub and Rabaptin 5, which compete for GAT binding (Mattera et al., 2004; Scott et al., 2004; Shiba et al., 2004). It has been shown previously by ITC that the heat generated by GGA3 GAT binding to Ub is contributed primarily by only one of the two known Ub-binding sites. This site, called site 1, is located upstream of the PI4P-binding residues identified here (Prag et al., 2005; Figure 2, A and B). Because site 1 dominates in this assay (Prag et al., 2005), our results suggest that PI4P promotes site 1 binding to Ub. Although not examined here, we speculate that site 2 might be modulated by PI4P as well, because the GGA1 PI4P-binding residue R265 is sandwiched between two critical site 2 Ub-binding residues (F264 and A267) that were identified by mutagenesis (Bilodeau et al., 2004; Mattera et al., 2004; Puertollano and Bonifacino, 2004; Akutsu et al., 2005; Kawasaki et al., 2005; Figure 2, A and B).

There has been much interest in the isoform-specific functions of the GGAs. Because GGA1 and GGA3 bind Ub with relatively low affinity, the approximately threefold increase in affinity by PI4P should facilitate their loading of the Ub cargoes at the TGN, which is enriched for PI4P. GGA2, which normally binds Ub even more weakly than GGA1 and GGA3 (Shiba et al., 2004; Puertollano and Bonifacino, 2004), is also activated by PI4P to bind Ub (Figure 5A). Therefore, GGA2 can potentially sort ubiquitinated cargoes when it is placed in a PI4P-rich environment of the TGN. The different inherent affinities/avidities of the various GGAs for Ub, and their common activation by PI4P may allow fine-tuned regulation of ubiquitinated versus nonubiquitinated cargo sorting at the TGN.

The mechanism for increasing the affinity of the GAT domain for Ub remains to be determined. The data presented here shows for the first time that there is a direct relation between phosphoinositides and the recognition of a Ub-sorting signal during membrane trafficking. Thus, the trafficking of ubiquitinated cargoes may be directly regulated by the same phosphoinositide that recruits their cognate adaptors to the appropriate organelle membranes. This, together with reports that phosphoinositides tether a ubiquitin ligase to internal organelle membranes (Dunn et al., 2004), and that GGA ubiquitination inhibits GGA binding to Ub cargoes (Hoeller et al., 2006; Yogosawa et al., 2006) underscores the emerging multifaceted roles of phosphoinositides in regulating membrane trafficking (Wenk and De Camilli, 2004).

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