PI(4)P-DEPENDENT RECRUITMENT OF CLATHRIN ADAPTORS TO THE TRANS-GOLGI NETWORK

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Dedicated to my parents

and my husband Kun Yang

PI(4)P-DEPENDENT RECRUITMENT OF CLATHRIN ADAPTORS TO THE TRANS-GOLGI NETWORK

by

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DISSERTATION

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Publication No. _____

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ABSTRACT

The Trans Golgi Network (TGN) is the cell's central sorting station, and the complex trafficking patterns are organized by many types of trafficking adaptors. These include the heterotetrameric adaptor protein complexes (APs) and the monomeric Golgi-localized, γ-ear containing, Arf-binding proteins (GGAs). The fundamental question of how these adaptors are recruited to TGN membrane remains unclear.

Previous studies have shown that adaptor recruitment to the TGN is absolutely dependent on the small GTPase ADP ribosylation factor 1 (Arf1), but paradoxically, Arf1 has a broader intracellular distribution than these adaptors. We found that the Golgi is particularly enriched in phosphatidylinositol 4 phosphate [PI(4)P] and that the clathrin adaptor AP-1 binds PI(4)P directly, suggesting that PI(4)P binding may specify the TGN-specific recruitment in conjunction with Arf1.

My studies showed that another monomeric clathrin adaptor GGA also binds PI(4)P and Arf1 independently. The C-terminal "triple helix bundle" of the GGA GAT domain is a polyfunctional module that interacts with multiple partners including PI(4)P and ubiquitin, and ubiquitin may provide a recognition signal for GGAs to control protein sorting. We found that PI(4)P increases wild type GAT binding to ubiquitin-conjugated agarose beads, but has no effect on a mutant GAT that does not bind PI(4)P. Therefore, PI(4)P may be an allosteric regulator of GGAs which enhances ubiquitin binding to GGAs.

Based on these results, we conclude: (1) PI(4)P defines the TGN organelle identity by recruiting TGN-targeted adaptors; (2) TGN-enriched adaptors are recruited to the Golgi by binding to both PI(4)P and Arf1, and neither alone is sufficient; (3) PI(4)P acts as a scaffold, and may also be an allosteric regulator for GGAs that modulates GGA function with other ligands.

We propose that the integration of combinatorial inputs from PI(4)P, Arf1 and ubiquitin may coordinately specify clathrin adaptor TGN recruitment through multiple low-affinity interactions.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AP:	adaptor protein complex
Arf:	ADP-ribosylation factor
CCVs:	clathrin coated vesicles
DAG:	diacylglycerol
EEA1:	early endosomal autoantigen 1
ER:	endoplasmic reticulum
FAPPs:	four-phosphate adaptor proteins
GAP:	GTPase activating protein
GAT:	GGA and TOM1 domain
GGA:	Golgi-localizing, γ-ear domain homolog, Arf-binding protein
GEF:	guanine nucleotide exchange factor
GFP:	green fluorescent protein
GST:	glutathione S-transferase
Hrs:	hepatocyte growth factor receptor tyrosine kinase substrate
MPR:	mannose 6-phosphate receptor
OSBP:	oxysterol binding protein
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PI4KIIα:	phosphatidylinositol 4 kinase type II α
PI(4)P:	phosphatidylinositol 4- phosphate
PIP2:	phosphatidylinositol 4,5-bisphosphate

PPIs: phosphoinositides

PS: phosphatidylserine

RNAi: RNA interference

siRNA: small interfering RNA

SPR: surface plasmon resonance

TGN: *trans*-Golgi network

Ub: ubiquitin

CHAPTER 1

Introduction

Part I: Membrane Traffic and Clathrin-Coated Vesicle Formation

1. Macro View of Membrane Traffic

Eukaryotic cells are highly compartmentalized into distinct membrane-bound organelles. Membrane traffic is the dynamic process responsible for biogenesis and organization of these organelles and for communication among them. It typically involves controlled formation of vesicles and vesiculo-tubular structures (collectively referred to as vesicles) from a donor membrane, directed movement of these vesicles to the target, and fusion with the acceptor membrane. These trafficking events must be tightly controlled in order to maintain the unique composition of individual organelles and prevent inappropriate mixing of compartments.

Secretion and endocytosis are two key routes by which a eukaryotic cell transports proteins (Figure 1) (van Vliet et al., 2003). Newly synthesized proteins are translocated into the endoplasmic reticulum (ER), then packed into COPII coated vesicles which fuse to become the ER-Golgi intermediate compartment (ERGIC). Multiple ERGICs merge to form the *cis*-Golgi network (CGN). ER proteins are packed into COPI coated vesicles and transported from the Golgi to ER in a retrograde manner. Anterograde cargo moves through the Golgi stacks and is sorted at the *trans*-Golgi network (TGN) to direct different types of coated vesicles transport cargo to multiple destinations. In the endocytic pathways, proteins are internalized at the plasma membrane and translocated to the early endosomes. Proteins at the early endosomes may be recycled to the cell surface, transported to the TGN or to the lysosome through the late endosome.

2. Molecular Mechanisms of Vesicular Transport

The idea of vesicular transport for membrane traffic was first brought out by early electron microscopy studies of cells (Roth and Porter, 1964). Now it is firmly established that membrane bound vesicles are commonly used in multiple transport steps in both the secretory and endocytic pathways. Although each step utilizes different proteins for coat formation, vesicle budding and fusion, the principles that are fundamental to these processes are quite similar.

The donor membrane is primed by the docking machinery, which includes a GTP-bound small G protein. Through various adaptors, the primed membrane recruits a polymeric coat that causes the membrane to invaginate and form a vesicle (Schekman and Orci, 1996). In some cases, additional proteins, such as dynamin, are required for final fission of the vesicle from the donor membrane (van der Bliek et al., 1993). Once the vesicle has detached from the membrane, GTP and/or ATP hydrolysis results in the dissociation of the coat. There are three well-defined vesicle coats, including COPI, COPII and clathrin coats. Each coat functions in a specific pathway or set of pathways to specify the transport of cargo from one membrane compartment to another (Figure 1).

Vesicle docking and fusion at the target membrane occurs in three steps (Pelham, 2001). First, the vesicle is linked to the target membrane by tethering complexes, which might be SNARE-associated, and regulated by Rab/ypt GTPases. Second, t-SNARE (on target membrane) and v-SNARE (on vesicle membrane) will interact to dock the vesicle onto the target membrane. Third, SNAREs form a tight complex to bring the membranes close together and initiate membrane fusion.

3. Clathrin and Clathrin Adaptor Proteins

Clathrin was the first membrane coat protein to be identified (Kanaseki and Kadota, 1969; Schmid, 1997). It has three heavy chains and three light chains that self-assemble into a triskelion, a structure with three bent legs radiating from a central point (Ungewickell and Branton, 1981). These clathrin triskelions assemble into polyhedral lattices, termed clathrin baskets or coats. Clathrin coated vesicles (CCVs) are found to be involved in various trafficking pathways including transport of proteins from the TGN to endosomes and from the plasma membrane to endosomes (Figure 1).

Clathrin typically associates with membranes via adaptor proteins, which also bind membrane cargo to act as a bridge linking clathrin to membranes. Different adaptors associate with different membranes through certain docking machinery, and pack specific cargoes into the correct CCVs. These multiple selections and interactions define a dynamic network to drive vesicle biogenesis at the right place and the right time.

Several large families of clathrin adaptors have been identified. These include the classical clathrin adaptors APs (adaptor protein complexes), which have four members with distinct functions (Ahle et al., 1988; Dell'Angelica et al., 1999; Robinson and Pearse, 1986; Simpson et al., 1997; Takatsu et al., 1998). In addition, there are two alternative members of clathrin adaptors. They are GGAs (Golgi-localizing, γ-ear-domain homology, Arf-binding proteins) at the TGN (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000) and Hrs (hepatocyte growth factor receptor tyrosine kinase substrate) on the endosome (Raiborg et al., 2002).

4. Clathrin-Coated Vesicle Formation

CCV genesis on the plasma member is extensively studied. Clathrin coats first associate with membrane through adaptor binding. Self polymerization of clathrin triskelions in conjunction with epsins result in curvature of the membrane at budding sites to form clathrin-coated pits (Ford et al., 2002; Marsh and McMahon, 1999). Recent live cell imaging studies by Kirchhausen and colleagues show that coated pits initiate randomly (Ehrlich et al., 2004). They may be stabilized by cargo capture, and continue to grow until membrane constricts to form a neck. Alternatively, coated pits will collapse rapidly if conditions required for coat stabilization are not met.

Fission of the vesicle from the donor membrane requires the activity of the GTPase dynamin (van der Bliek et al., 1993). Dynamin self-assembles into a collar of helices around the neck of the invaginated membrane, and GTP hydrolysis allows dynamin to pinch the coated vesicle from the membrane (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998). Following budding, auxillin binds to the clathrin coat and recruits the chaperone heat shock cognate 70 (Hsc70) which catalyses the disassembly of the clathrin coat coupled to ATP hydrolysis (Ungewickell et al., 1995).

5. TGN Membrane Recruitment of Clathrin Adaptors

TGN is the central sorting station of the cell, and its complex trafficking patterns are organized by many types of adaptors. The fundamental question of how these adaptors are recruited to TGN membrane remains unclear.

Previous studies show that binding of adaptors to membranes requires activation of the GTPase ADP ribosylation factor 1 (Arf1). There are six Arf proteins, and they can be grouped

into three families according to their sequence homology (Spang, 2002). Arfs 1, 2, and 3 are found at the Golgi (as well as elsewhere in the cell) where they regulate the formation of COPI and clathrin-coated vesicles and are required for many secretory events (Donaldson et al.; Lee et al., 2005; Stamnes and Rothman, 1993; Traub et al., 1993). Arf6 functions at the plasma membrane to regulate actin dynamics and vesicular trafficking (Krauss et al., 2003; Radhakrishna and Donaldson, 1997; Radhakrishna et al., 1996; Song et al., 1998; Tanabe et al., 2005). The role of Arf4 or 5 has not been reported.

Arfs' membrane association and dissociation are mainly regulated by a number of the guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Nie et al., 2003). GEFs exchange Arf-bound GDP with GTP, and activate Arfs from their soluble GDP-bound form to the membrane-associated GTP-bound form. GAPs promote the hydrolysis of Arf-bound GTP to GDP. Myristoylation are also important for Arf activation (Franco et al., 1995). Myristolated Arf1 favors GDP dissociation and has higher affinity for liposome.

Arf1 is a major regulator of membrane traffic at the Golgi. TGN membrane recruitment of clathrin adaptors depends on Arf1 in its GTP-bound state (Collins et al., 2003; Puertollano et al., 2001; Stamnes and Rothman, 1993; Traub et al., 1993). However, although Arf1-GTP is absolutely required for adaptors TGN recruitment, paradoxically, it has a broader intracellular distribution than these adaptors. Therefore, it has long been hypothesized that additional docking site(s) are required to specify the TGN membrane recruitment of clathrin adaptors (Zhu et al., 1999). Now our lab found that a second factor is phosphatidylinositol 4 phosphate [PI(4)P]. We propose that PI(4)P is another essential docking site, and that both PI(4)P and Arf1 are necessary for the recruitment of cytosolic adaptor protein recruitment to the TGN. The rationale and evidence will be described below.

Part II: A Role of Phosphatidylinositol 4 Phosphate in TGN Functions

1. Phosphoinositides Overview

Phospholipids are important building blocks for cell membranes. They are essential for both cell structure and cell metabolism. There are more than 200 different phospholipid species identified in the average cell membrane. Among them, phosphoinositides [PPIs, which collectively refer to phosphatidylinositol (PI) and its phosphorylated derivatives] have received much attention recently. Numerous physiological functions depend on the regulated turnover of PPIs, which are spatially and temporally controlled via phosphorylation/dephosphorylation reactions by specific kinases and phosphatases (Figure 2) (Clarke, 2003).

The importance of phospholipid turnover was first recognized more than 50 years ago by Lowell and Mabel Hokin (Hokin and Hokin, 1953), who showed that cholinergic stimulation of pancreatic secretion is associated with phosphorylation of membrane phospholipids. Subsequently it became apparent that only the phosphoinositides are involved in this stimulated turnover. Moreover, although PPIs accounts for only a small fraction (5-10%) of the total membrane lipids, their regulated turnover occurs in a wide variety of physiological processes, such as membrane trafficking and cytoskeleton rearrangement that underlies cell proliferation and growth (Yin and Janmey, 2003). In all these different functions, PPIs serve as both structural and signaling molecules.

2. PPIs Are Involved in Membrane Traffic

PPIs have been implicated in many membrane trafficking events. Primarily they are scaffolds for the recruitment of peripheral membrane proteins that are involved in sorting protein

cargos or for the docking and fusion of transport vesicles (Cremona and De Camilli, 2001; De Camilli et al., 1996; Martin, 2001). In some cases, the activities of these proteins are modified when they bind a particular PPI (Barylko et al., 2001a), indicating that these lipids can also act as allosteric regulators.

Different PPIs are concentrated in different parts of the membrane trafficking pathway. Phosphatidylinositol 4,5 bisphosphate [PI(4,5)P2 or PIP2] is primarily at the plasma membrane (Brown et al., 2001; Holz and Axelrod, 2002), phosphatidylinositol 3 phosphate [PI(3)P] is the major phosphatidylinositide on early endosomes (Burd and Emr, 1998; Ellson et al., 2001), and phosphatidylinositol 3,5 bisphosphate [PI(3,5)P2] is found on late endocytic organelles (Whiteford et al., 1997) (Figure 3) (Roth, 2004; Wang et al., 2003). Our lab established that phosphatidylinositol 4 phosphate [PI(4)P] is predominant at the TGN (Wang et al., 2003). The spatial segregation of PPIs may be a mechanism by which the direction of membrane traffic and the specificity of the cargo-vesicle selection are controlled.

3. PI(4)P Regulates Some Golgi Functions Independently of Downstream Generation of PIP2

PIP2 is now firmly established as an essential regulator of plasma membrane trafficking (Cremona and De Camilli, 2001), and has also been implicated in the trafficking of other organelle membranes (Brown et al., 2001; Rozelle et al., 2000), including the Golgi (Cockcroft and De Matteis, 2001; De Matteis et al., 2002). However, in spite of the overwhelming evidence for PIP2 regulation of plasma membrane trafficking, a direct role of PIP2 in Golgi membrane trafficking has not been established. Strikingly, all currently available evidence suggests that the Golgi, unlike the plasma membrane, has remarkably little PIP2. Watt et al (Watt et al., 2002) used electron microscopy and a PLCδ1-PH domain probe to quantify PIP2 on cellular

membranes. They found that the highest density of labeling was at the plasma membrane, and the Golgi has nine fold lower PIP2 density than the plasma membrane does. By immunofluorescence staining with specific antibodies, our lab showed that PI(4)P, the immediate precursor of PIP2, was concentrated on Golgi, in striking contrast to PIP2, which is predominantly at the plasma membrane (Wang et al., 2003). Thus, PI(4)P, and not PIP2, is the major phosphoinositide on mammalian Golgi membranes, and the pool of free PIP2 on the Golgi is relatively small.

The low abundance of PIP2 on Golgi membranes suggests that it is unlikely to be involved in the stoichiometric recruitment of the large assortment of coat proteins onto the TGN. PI(4)P, however, could potentially fulfill this role because it is more abundant than PIP2 at the TGN, and several TGN-associated proteins that bind PI(4)P have recently been identified. These include AP-1 (Wang et al., 2003), GGAs (my unpublished data), epsinR (an AP-1 accessory protein) (Hirst et al., 2003; Mills et al., 2003), oxysterol binding protein (OSBP) (Levine and Munro, 2002), and four phosphate adaptor proteins (FAPPs) (Godi et al., 2004). It was also discovered that *S. cerevisiae* uses PI(4)P, but not PIP2, to regulate constitutive secretion from the late Golgi (Audhya et al., 2000; Hama et al., 1999; Walch-Solimena and Novick, 1999). Therefore, PI(4)P is beginning to emerge as a Golgi regulator in its own right.

4. Regulation of Golgi PI(4)P

To understand further the spatial and temporal regulation of PI(4)P, it becomes necessary to know which of the numerous phosphoinositide kinases and phosphatases are involved, and how they are regulated.

Lipid Kinases

Two distinct classes of kinases phosphorylate PI at the *D*-4 position to generate PI(4)P. These are called type II and type III phosphatidylinositol 4 kinase (PI4K) (Table 1; Figure 4). Type I was discovered to be a PI 3-kinase and will not be discussed here.

In yeast, the Golgi PI(4)P is generated by a Golgi-associated type III PI4K called Pik1p (Flanagan et al., 1993; Walch-Solimena and Novick, 1999). The other major type III PI4K, Stt4p, is important for cell wall integrity and cytoskeleton rearrangements (Audhya et al., 2000). It has no known role in secretion. The yeast type II PI4K, called LSB6, does not appear to have an essential role (Han et al., 2002; Shelton et al., 2003).

Mammals have the same two type III PI4K orthologs as yeast (called PI4KIIIα and PI4KIIIβ or PI4Kβ) and two type II PI4Ks called PI4KIIα and β (Balla et al., 2002; Barylko et al., 2001b; Minogue et al., 2001; Wei et al., 2002). In contrast to yeast, we found that PI4KIIα in the mammal has an important role in TGN trafficking. It is highly enriched in secretory vesicles of neuronal cells (Guo et al., 2003). In non-neuronal cells, it is primarily TGN associated. It behaves as an integral membrane protein, although it does not have a recognizable transmembrane domain (Wei et al., 2002). Cytosolic PI4KIIIβ is recruited to Golgi enriched membranes in an Arf1 dependent manner together with PIP5Ks that convert PI(4)P to PIP2 (De Matteis et al., 2002; Godi et al., 1999). PI4KIIβ is predominantly cytosolic, but is associated with the plasma membrane and the Golgi as a peripheral protein (Wei et al., 2002). PI4KIIIα was originally reported to be ER-and Golgi-associated (Aikawa et al., 1999), although more recent studies report that it is primarily located in the plasma membrane (Balla et al., 2005).

To summarize, at least three of the mammalian PI4Ks have been definitively localized to the Golgi (Godi et al., 1999; Wei et al., 2002; Wong and Cantley, 1997), either as an integral

protein or a peripheral protein. The challenge is to determine their relative contributions to TGN functions.

To answer this question, our lab, and subsequently other labs, used the RNA interference (RNAi) strategy to assess their differential functions. PI4KIIα RNAi decreased the PI4KIIα protein level in HeLa cells by approximately 80%. This was accompanied by a 60% decrease in PIP and 50% decrease in PIP2 (Wang et al., 2003). These cells had decreased staining of several PI(4)P-dependent Golgi proteins (AP-1, GGAs, epsinR, FAPP1 and OSBP) (Balla et al., 2005; Wang et al., 2003), suggesting that PI4KIIα is the dominant PI4K in the Golgi and maintains a constitutive Golgi PI(4)P pool. Its function in membrane trafficking will be discussed in detail in the following chapters. In contrast, PI4KIIβ RNAi does not generate detectable changes in the Golgi (Wang et al., 2003), although it alters the actin cytoskeleton (our unpublished data). PI4KIIIβ RNAi does not affect clathrin adaptors (AP-1 and GGAs) association with the TGN membrane (Wang et al., 2003). However, PI4KIIIβ knockdown decreases immunofluorescence staining of a TGN resident protein TGN46 (Wang et al., 2003). This phenotype is probably due to abnormal trafficking of this transmembrane protein, or a generalized loss of perinuclear Golgi material. In summary, these results imply that the three PI4Ks have distinct roles in the regulation of Golgi functions.

Lipid Phosphatases

The size of the PI(4)P pool can also be regulated by lipid phosphatases. There is very little information on inositol polyphosphate 4-phosphatase (4-phosphatase) that removes the 4-phosphate from the inositol ring. More is known about inositol polyphosphate 5-phosphatases (5-phosphatase), which hydrolyzes the 5-phosphate off a group of substrates including PI(4,5)P2. Cells use 5-phosphatase to decrease the PI(4,5)P2 pool, and theoretically, to produce PI(4)P.

Two members of 5-phosphatases are extensively studied. These are synaptojanin, which plays key roles in the clathrin-mediated endocytosis of synaptic vesicles on the plasma membrane (Chuang et al., 2004; Ramjaun and McPherson, 1996), and Ocrl (Ungewickell et al., 2004).

Ocrl is mutated in patients with Lowe syndromes (Zhang et al., 1998). It was reported recently that Ocrl colocalizes with clathrin, the mannose 6-phosphate receptor (MPR), transferrin, and the early endosomal autoantigen 1 (EEA1) on the endosomes and Golgi membranes (Ungewickell et al., 2004). Ocrl also interacts with clathrin and clathrin adaptor protein complex 2 (AP-2). Their findings suggest that Ocrl may have a role in endosomal receptor trafficking and sorting at Golgi and early endosomes, either by regulating PIP2 homeostasis or by generating PI(4)P from PIP2.

Part III: Objective and Organization of the Dissertation

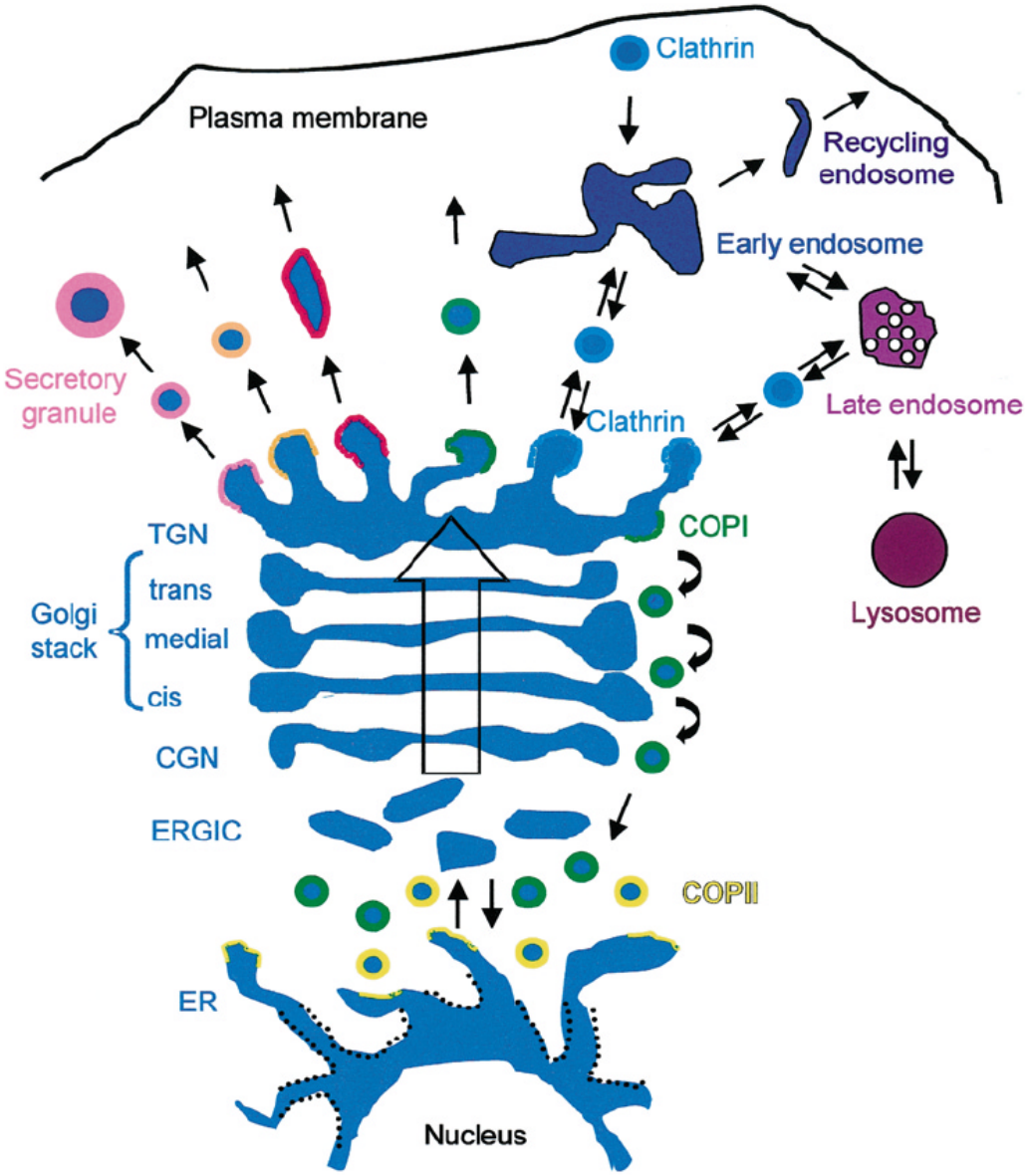
The overall objective of this dissertation is to establish that PI(4)P is a *bona fide* TGN marker, and that it defines the TGN's lipid organelle identity. Clathrin adaptors are recruited to the TGN by binding PI(4)P in conjunction with Arf1-GTP. PI(4)P and Arf1 are components of a "coincidence detection network" that specify recruitment of adaptors to the TGN through multiple low affinity interactions. I tested this hypothesis with two major clathrin adaptor families, the adaptor protein complex-1 (AP-1) and the GGAs.

Chapter 2 establishes that AP-1 recruitment to the TGN is mediated through PI(4)P binding. We found that PI(4)P is enriched at the TGN, and PI4KIIα is the major PI4K to generate this PI(4)P pool. We used PI4KIIα RNAi to knock down PI4KIIα specifically and found that recruitment of clathrin adaptor AP-1 complex to the Golgi was blocked. Independent

biochemical studies show that there is a direct binding between AP-1 and PI(4)P. This interaction is further characterized by crystallography and mutagenesis studies in **Chapter 3**. We identified key PI(4)P-binding residues in gamma chain of AP-1 complex.

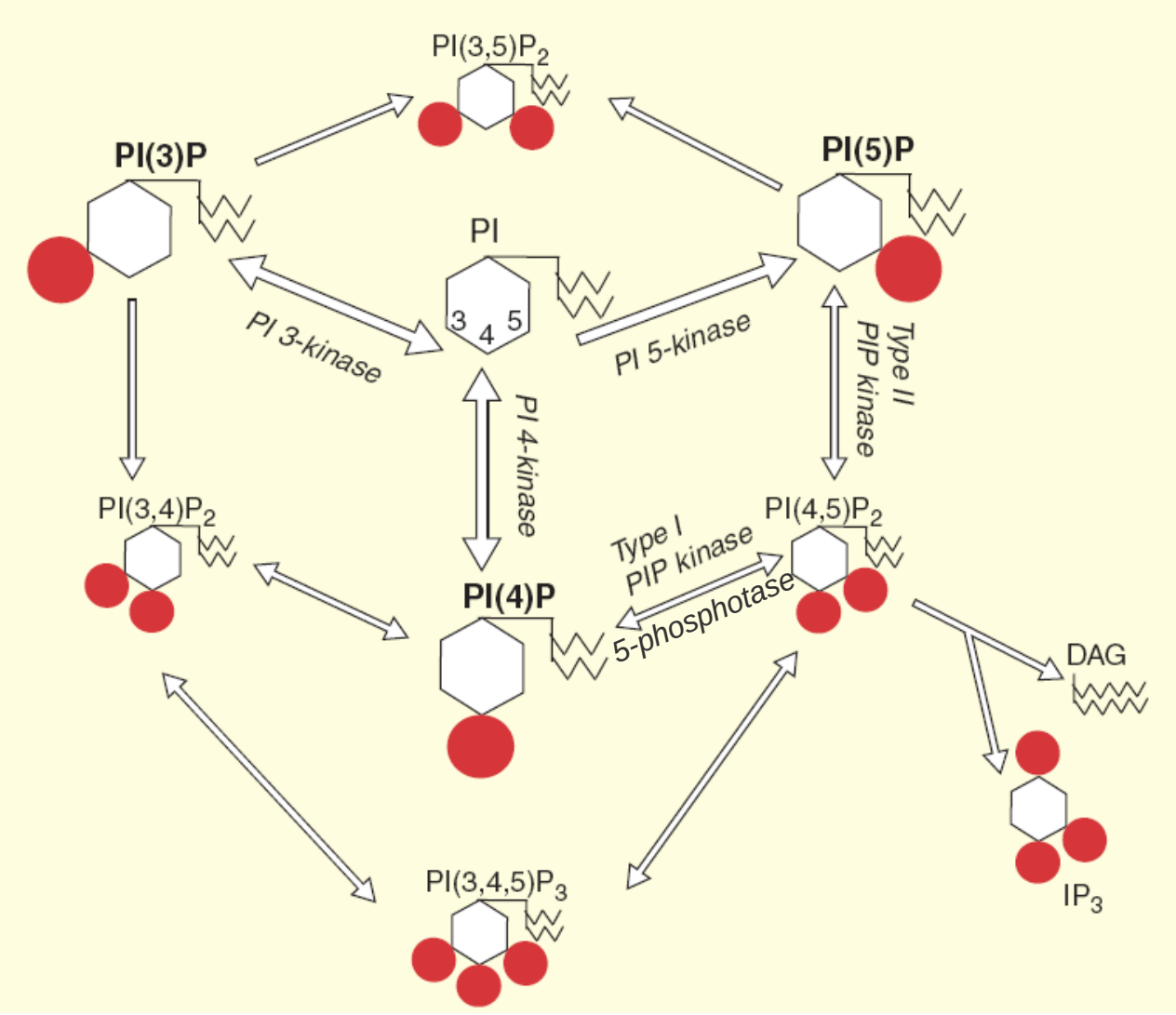
Chapter 4 shows that the GGA family of clathrin adaptors also bind to PI(4)P. Knockdown of PI4KIIα decreased GGAs recruitment to the TGN. Similarly to AP-1, we proposed a direct binding between GGAs and PI(4)P. Using a series of deletion experiments, I located the PI(4)P binding domain at the C-terminal "triple helix bundle" of the GAT domain. Site-directed mutagenesis showed that two residues in this region are important for GGA PI(4)P interaction. We also found that PI(4)P promotes GAT binding to ubiquitin. Therefore, PI(4)P may have dual roles on GGAs functions at the TGN. It is a scaffold for GGAs TGN recruitment in conjunction with Arf1, and it may also be an allosteric regulator for GGAs to enhance ubiquitin binding to GGAs.

Chapter 5 summarizes the thesis and provides current perspectives. We will make more efforts on quantitative analysis of GGA GAT interaction with ubiquitin in the presence of PI(4)P.



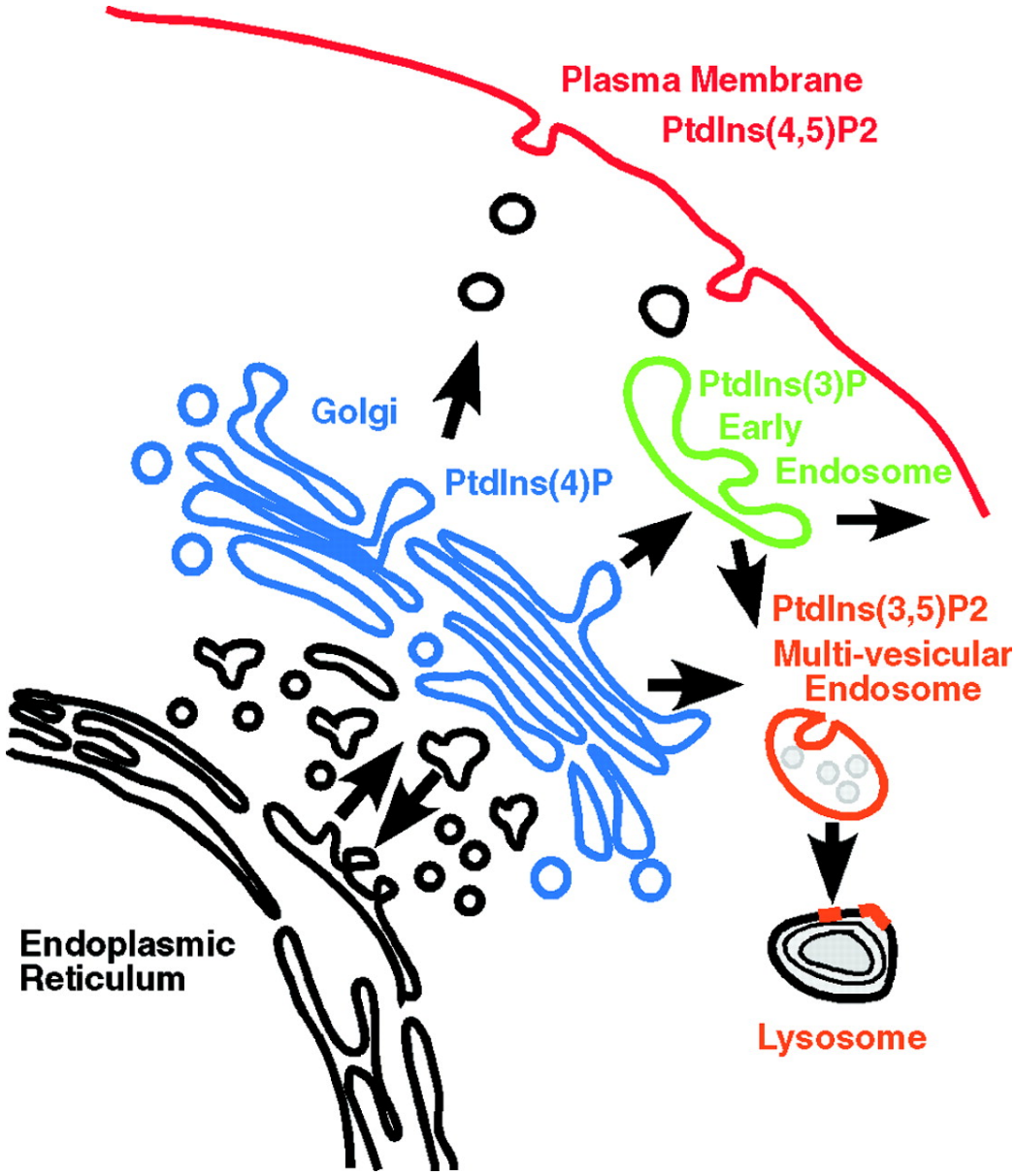
(van Vliet, 2003)

Figure 1. The secretory and endocytic pathways (adapted from *van Vliet, 2003*). Newly synthesized proteins are translocated into the endoplasmic reticulum (ER), then packed into COPII coated vesicles which fuse to become the ER-Golgi intermediate compartment (ERGIC). Multiple ERGICs merge to form the *cis*-Golgi network (CGN). ER proteins are packed into COPI coated vesicles and transport from the Golgi to ER in a retrograde manner. Anterograde cargo moves through the Golgi stacks and is sorted at the *trans*-Golgi network (TGN), to direct different types of coated vesicles transport cargo to multiple destinations. In the endocytic pathways, proteins are internalized at the plasma membrane and translocated to the early endosomes. Proteins at the early endosomes may be recycled to the cell surface, transported to the lysosome through the late endosome or to the TGN.


PI(3,5)P2
PI(3)P
PI(5)P
PI
3 4 5
PI 3-kinase
PI 5-kinase
Type II
PIP kinase
PI 4-kinase
PI(3,4)P2
PI(4,5)P2
Type I
PIP kinase
PI(4)P
5-phosphotase
DAG
IP3
PI(3,4,5)P3

(Clarke, 2003)

Figure 2. The synthesis and interconversion of PPIs (adapted from *Clarke, 2003*). PPIs undergo phosphorylation/dephosphorylation reactions catalyzed by kinases and phosphatases. PI can be converted to mono-phosphorylated PPIs by specific kinases as indicated. PI(4,5)P2 is produced mainly from PI(4)P by type I PIP kinases. Inositol polyphosphate 5-phosphatase (5-phosphatase) acts on PI(4,5)P2 to remove a phosphate from *D*-5 position of the inositol ring. PI(4,5)P2 can also be hydrolyzed by phospholipase C to generate second messengers IP3 and diacylglycerol (DAG).


Plasma Membrane
PtdIns(4,5)P2
Golgi
PtdIns(4)P
PtdIns(3)P
Early
Endosome
PtdIns(3,5)P2
Multi-vesicular
Endosome
Endoplasmic
Reticulum
Lysosome

(Roth, 2004)

Figure 3. Compartmentalization of PPIs (adapted from *Roth, 2004*)**.** Some PPIs are concentrated at distinct sites in intracellular membrane traffic pathways and may serve as organelle markers. PI(4)P (blue) is concentrated at the Golgi complex. PI(3)P (green) is concentrated on early endosomes. The majority of PI(4,5)P2 (red) is at the plasma membrane at steady state. PI(3,5)P2 (orange) is found on multivesicular endosomes and lysosomes.

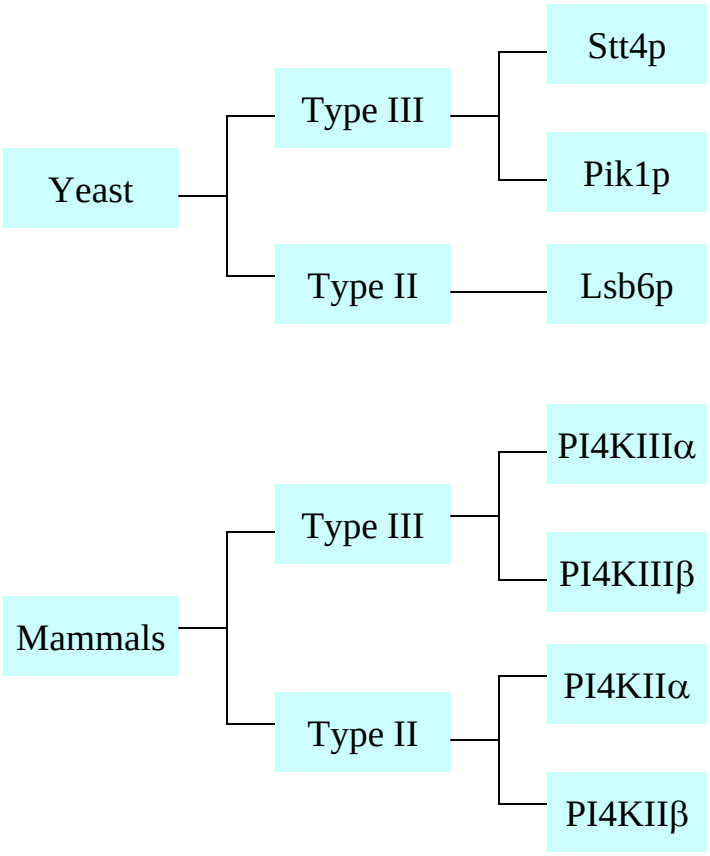

Yeast
Type III
Stt4p
Pik1p
Type II
Lsb6p
Mammals
Type III
PI4KIIIα
PI4KIIIβ
Type II
PI4KIIα
PI4KIIβ

Figure 4. PI4Ks in yeast and mammalian cells. PI4Ks are grouped into two distinct classes: type II and type III (type I was discovered to be a PI 3-kinase). Yeast has two type III PI4Ks. Stt4p is important for cell wall integrity and cytoskeleton rearrangements. Pik1p is Golgi associated. It generates Golgi PI(4)P and is essential for normal secretion. The type II PI4K in yeast is called Lsb6p. Mammals have the same two type III PI4K orthologs as yeast (called PI4KIIIα and PI4KIIIβ) and two type II PI4Ks called PI4KIIα and β. PI4KIIIα (equivalent to yeast Stt4p) was originally reported to be ER-and Golgi-associated, although more recent studies report that it is primarily located in the plasma membrane. PI4KIIIβ (equivalent to yeast Pik1p) is cytosolic. It is recruited to Golgi enriched membranes in an Arf1 dependent manner together with PIP5Ks that convert PI(4)P to PIP2. PI4KIIα is the major kinase to generate PI(4)P at the TGN in non-neuronal cells. PI4KIIβ is predominantly cytosolic, but associates with the plasma membrane and the Golgi as a peripheral protein.

Table 1. Enzymatic and Biochemical Properties of PI4Ks

	Type II	Type III
Km value for PI and ATP	1x	3~7x
Sensitivity to adenosine	~20x	1x
Sensitivity to wortmannin	insensitive	sensitive
Molecular weight	~55KDa	>100KDa

CHAPTER 2

Phosphatidylinositol 4 Phosphate Regulates Targeting of the Clathrin Adaptor AP-1 Complexes to the Golgi

Introduction

Functions of AP-1

The name "AP" was originally introduced as an acronym of "assembly polypeptides", which have the capacity to bind clathrin and assemble it into coats (Keen et al., 1979), and now conveniently it also stands for "adaptor protein". AP-1 and AP-2 were the first two coated vesicle adaptors to be identified (Ahle et al., 1988; Robinson and Pearse, 1986). They are heterotetramers and contain related sets of subunits (γ, β1, μ1 and σ1 in AP-1;α, β2, μ2, and σ2 in AP-2) (Figure 5). They are both highly enriched in purified clathrin-coated vesicles, but they localize to different membranes: AP-1 is found on the TGN and endosomes, whereas AP-2 is found at the plasma membrane. Two additional adaptor complexes, AP-3 and AP-4, were discovered recently. Like AP-1, they are also found on TGN/endosomal membranes, with AP-3 localized more to endosomes and AP-4 more to the TGN (Dell'Angelica et al., 1999; Simpson et al., 1997). The question of how they generate distinct transport vesicles from a common Golgi membrane is not well understood (Bonifacino and Lippincott-Schwartz, 2003).

There are two AP-1 variants, AP-1A and AP-1B (Folsch et al., 1999; Folsch et al., 2003). AP-1A is ubiquitously expressed and is involved in membrane traffic between TGN and endosomes. AP-1B is only expressed in epithelia cells. It mediates protein targeting to the basolateral surface of the plasma membrane. Both AP-1 complexes are heterotetramers and differ slightly only in their 50-kD mu1A or mu1B subunits (Folsch et al., 1999). AP-1 plays a

central role in the sorting and packaging of membrane proteins into Golgi derived CCVs. These vesicles transport two classes of mannose 6-phosphate receptors (cation dependent and independent MPRs) with bound lysosomal enzymes from the TGN to endosomes (anterograde), and retrieve them from endosomes back to the TGN (retrograde) (Meyer et al., 2000).

The importance of AP-1 is established by knockout of the μ1 subunit of AP-1A with homologous recombination. μ1-/- is embryonic lethal, and the μ1 deficient fibroblasts have defective MPR trafficking which can be rescued by overexpressing μ1 (Meyer et al., 2001). This phenotype is most consistent with the inability to retrieve MPRs from endosomes to the TGN, rather than in anterograde transport. Nevertheless, recent studies suggest that AP-1 is important in anterograde transport as well, most likely by cooperating with the GGAs (Golgi associated, γ-ear containing, Arf-binding proteins) (Doray et al., 2002).

Analogy of AP-2 to AP-1

While AP-2 is similar to AP-1 structurally (Figure 5), it is involved in CCV generation at the plasma membrane instead of the TGN. More is known about the role of the PIP2 in regulating AP-2 functions (Collins et al., 2002). AP-2 has two PIP2/PIP3 binding sites located in its α and μ2 subunits. Its crystal structure suggests a *two-step model* for phosphoinositide dependent recruitment and activation. In the absence of PIP2, AP-2 exists in a *closed* conformation in which μ2 subunit is sequestered within the adaptor core. The α-adaptin site first targets AP-2 to the plasma membrane by binding PIP2/PIP3. This induces a large conformational change that flips the μ2 subunit out of the adaptor core (*open* conformation) to expose the second PIP2 binding site and an adjacent cargo binding site on the surface of μ2.

Since unlike the plasma membrane, the TGN has very little PIP2 (Watt et al., 2002), PIP2 is unlikely to be the docking site for AP-1 at the TGN. Sequence analysis also reveals that AP-1's putative phosphoinositide binding subunits (γ and μ1) lack a few of the basic residues that contact PIP2 in AP-2. Therefore, AP-1 is predicted to prefer less highly charged phosphoinositides [such as PI(4)P instead of PIP2] (Collins et al., 2002).

In this study, we show that PI(4)P is enriched at the TGN, and AP-1 adaptor complex directly binds PI(4)P. Furthermore, PI(4)P, but not PIP2, is required for AP-1 recruitment to the TGN, and this PI(4)P is generated primarily by the Golgi resident kinase PI4KIIα.

Results

siRNA Knocks Down PI4KIIα Expression

We used RNA interference (RNAi) (Elbashir et al., 2001) to examine PI4KIIα's role in the mammalian Golgi. Western blotting showed that PI4KIIα protein expression was reduced to 76% of control level at 72 hr after exposure to siRNA (small interfering RNA) (Figure 6A). The decrease was specific for PI4KIIα; PI4KIIIβ, PI4KIIβ, human type I phosphatidylinositol phosphate 5 kinase α (PIP5KIα), and actin levels were not significantly changed.

Immunofluorescence staining confirmed that approximately 80% of PI4KIIα siRNA-treated cells had no or reduced PI4KIIα staining (Figure 6C). Nevertheless, most of these cells had perinuclear TGN46 staining, at intensities that were comparable to that of control cells (Table 2). Therefore, PI4KIIα RNAi did not grossly disrupt the Golgi under these conditions. The TGN46 staining pattern was however altered in appearance. Its mean area increased 3.1-fold (n = 10) and staining was more punctuated and/or tubular compared with control cells. The majority of cells with decreased PI4KIIα expression exhibited the expanded Golgi phenotype.

PI4KIIα RNAi Inhibits Phosphoinositide Synthesis and Selectively Decreases Golgi PI(4)P

PI4KIIα siRNA profoundly inhibited ^{32}P incorporation into PI(4)P and PIP2 (to 48.9 ± 6.3% and 58.9 ± 14.4% of control, respectively [n = 5]) (Figure 6B). PI4KIIα is therefore a major PI4K and a significant contributor to overall PI(4)P and PIP2 synthesis.

We used anti-PI(4)P and a GFP-PH reporter to visualize PI(4)P pools in cells. We avoided using detergents in order to maximize the preservation of membrane lipids. Cells were fixed with formaldehyde and then subjected to one controlled freeze-thaw cycle in the presence of 1 M sucrose (Tran et al., 1999). In control cells, PI(4)P immunofluorescence was enriched in the Golgi and in a band of cytoplasm surrounding the nucleus (Figure 6C). The Golgi PI(4)P fluorescence accounts for 46.5% of total PI(4)P (Table 2). Surprisingly, the plasma membrane had very little anti-PI(4)P staining (Figure 6C). PI4KIIα RNAi dramatically reduced the intensity of Golgi PI(4)P fluorescence (Figure 6C), which now accounts for only 16.7% of total PI(4)P, instead of 46.5% in control cells (Table 2). Based on these values, we estimate that roughly 36% (16.7 divided by 46.5) of PI(4)P remains in the Golgi after RNAi. This is likely to be an underestimate, because we did not take into account the PI(4)P decrease in other regions of the cell.

The decrease in Golgi PI(4)P was confirmed by using OSBP-PH as a PI(4)P reporter (Levine and Munro, 2002) (Figure 6C). Overexpressed GFP-OSBP-PH was no longer concentrated in the perinuclear Golgi in PI4KIIα RNAi cells.

PI4KIIα RNAi Blocks AP-1 Association With the TGN

PI4KIIα RNAi dramatically decreased the association of γ-adaptin (a subunit of the AP-1 complex) with the Golgi, both in terms of the percentage of cells with strong perinuclear γ-

adaptin (89% versus 29%) and their intensity (12.1 A.U. to 3.6 A.U.) (Figure 7A, Table 2). In contrast, β-COP (a component of the COP1 coatomer complex) remained on the Golgi, although it was also expanded because the Golgi is enlarged (Figure 7B).

The Golgi has two other PI4Ks. We therefore used RNAi to determine if they are also required for AP-1 recruitment. PI4KIIβ RNAi had no obvious effect on either γ -adaptin or TGN46 perinuclear staining (Figure 7A). PI4KIIIβ decreased Golgi γ-adaptin and TGN46 intensities in parallel in most cells, suggesting that the decrease may be secondary to the loss of Golgi membranes. Additional studies will be required to determine if PI4KIIIβ RNAi disrupts the Golgi. From the results presented here, it is clear that PI4KIIα RNAi uniquely blocks AP-1 association with the Golgi.

AP-1 regulates CCV trafficking between the TGN and endosome/lysosme system (Bonifacino and Lippincott-Schwartz, 2003). As expected from the profound loss of AP-1 in PI4KIIα RNAi cells, Golgi clathrin is also greatly reduced (Figure 7B). In addition, mannose 6 phosphate receptors (MPR300) are scattered throughout the cytoplasm, instead of clustering normally around the TGN (Figure 7B). This recapitulates the MPR mislocalization phenotype observed in $\mu 1^{-/-}$ fibroblasts that do not have functional AP-1 complexes (Meyer et al., 2001; Meyer et al., 2000).

AP-1 Directly Binds PI(4)P

PI4KIIα synthesizes PI(4)P, and PI(4)P is the immediate precursor of PIP2. Therefore, the decrease in Golgi AP-1 after PI4KIIα RNAi is likely to be a direct consequence of the loss of either Golgi PI(4)P or PIP2. We used a number of approaches to determine whether PI(4)P or PIP2 is the regulator of AP-1 Golgi recruitment.

As a first step, we examined the possibility that AP-1 directly binds phosphoinositides. Using a solid phase lipid binding assay, we found that purified AP-1 binds PI(4)P slightly better than PI(5)P. Since there is much less PI(5)P than PI(4)P in cells (49 times less according to one estimate) (Rameh et al., 1997a; Rameh et al., 1997b), and PI(4)P is most concentrated in the Golgi (Figure 6C), PI(4)P is likely to be the primary lipid for AP-1 recruitment.

In addition, AP-1 bound phosphatidic acid (PA) (Figure 8A). We do not know if this interaction is physiologically relevant, because AP-1 did not bind PA coupled beads (Krugmann et al., 2002).

Most significantly, AP-1 did not bind PI(4,5)P2 and PI(3,4,5)P3 (Figure 8), establishing that it has a different lipid specificity than the plasma membrane-associated AP-2 adaptor protein (Gaidarov and Keen, 1999; Rohde et al., 2002). AP-1's preference for less highly charged phosphoinositides [such as PI(4)P instead of PIP2] was in fact predicted based on the fact that AP-1's putative phosphoinositide binding subunits (γ and μ1) lack a few of the basic residues that contact PIP2 in AP-2 (Collins et al., 2002).

AP-1 Association with the Golgi is Regulated by PI(4)P

Having demonstrated that AP-1 has the potential to bind PI(4)P, we wanted to determine if PI(4)P is required for AP-1 recruitment to the Golgi. Three approaches were used. First, we attempted an *in vivo* PI4KIIα RNAi rescue experiment. PI(4)P or PIP2 was shuttled into live cells with membrane permeate polyamine carriers (Ozaki et al., 2000). After incubation with shuttle PI(4)P for 30 min at 37°C, at least 79% of PI4KIIα RNAi cells had perinuclear γ-adaptin staining compared with 29% of cells incubated with carrier alone (Figure 9A, Table 2). The average perinuclear γ-adaptin intensity increased from 3.6 A.U. to 7.0 A.U., which is 29.8% and

57.9%, respectively, of control cells without PI4KIIα RNAi (Table 2). Carrier alone or lipid without carrier had no effect, establishing that PI(4)P rescued AP-1 recruitment after it entered cells.

The brief diC16-PI(4)P treatment did not reverse the expanded Golgi morphology (Figure 9A), perhaps because Golgi reorganization requires more time than simple restoration of AP-1 binding. In contrast, shuttled PIP2 did not rescue AP-1 binding (Figure 9A, Table 2). In fact, it consistently decreased the percent of cells with Golgi AP-1 (Table 2). The basis for this was not explored further, but it is clear that PIP2 does not promote AP-1 recruitment.

In a second series of experiments, we studied AP-1 recruitment in semi-intact cells. As expected, cytosolic γ-adaptin was recruited to the Golgi of control cells, resulting in a 2.5-fold increase in γ-adaptin intensity (Figure 9B, Table 3). In contrast, the Golgi of PI4KIIα RNAi cells, which had less Golgi γ-adaptin staining initially, were still not able to recruit AP-1 from the normal control cytosol (Table 3). These results showed that the primary defect is inherent in the PI4KIIα RNAi Golgi and not due to depletion of a cytosolic recruiting factor by RNAi. This defect was overcome by adding exogenous PI(4)P, resulting in a 2.3-fold increase in bound γ-adaptin (Figure 9B, Table 3). PIP2 again had no effect. Therefore, defective AP-1 binding is due to a lack of Golgi PI(4)P but not PIP2.

In the third series of experiments, we examined the impact of reducing PI(4)P accessibility on AP-1 recruitment to normal membranes. Anti-PI(4)P pretreatment of microsome membranes blocked AP-1 binding, while anti-PIP2 and anti-talin (used as a control here) did not (Figure 9C). Thus, anti-PI(4)P selectively inhibits AP-1 recruitment, establishing that PI(4)P is critically important and that it acts independently of PIP2.

Discussion

We have identified PI4KIIα as the major Golgi resident PI4K that generates a large portion of total cellular PI(4)P and PIP2, and particularly Golgi PI(4)P. We achieved significant PI4KIIα RNAi knockdown without affecting the expression of several other closely related lipid kinases. The observed phenotypes were therefore due to a *bona fide* loss-of-function.

PI(4)P-Dependent AP-1 Recruitment to the Golgi

PI4KIIα RNAi decreased the amount of Golgi PI(4)P. It also decreased the association of AP-1 with the TGN, while PI4KIIβ or PI4KIIIβ RNAi did not. Therefore, PI4KIIα has a unique role in the Golgi.

We used a variety of methods to establish that PI(4)P is required for AP-1 recruitment *in vivo*. We find that shuttling PI(4)P into live cells or adding PI(4)P to semi-intact PI4KIIα RNAi cells restores AP-1 recruitment. Furthermore, blocking access to PI(4)P by anti-PI(4)P decreases AP-1 recruitment to normal membranes. Our results establish the following: (1) there is a cause-and-effect relation between the change in TGN PI(4)P and AP-1; (2) the interaction of AP-1 with the TGN is absolutely dependent on PI(4)P; (3) PIP2, which is important for plasma membrane recruitment of AP-2, is not involved in AP-1 recruitment; and (4) AP-1 directly binds PI(4)P but not PIP2.

Taken together, our results can be most simply explained by postulating that AP-1 binds the TGN by docking on TGN membrane PI(4)Ps.

The Relation between Arf1 and PI4KIIα in AP-1 Recruitment

Besides a requirement for PI(4)P shown in this paper, others have shown that AP-1 recruitment is also absolutely dependent on Arf1 (Bonifacino and Lippincott-Schwartz, 2003; Zhu et al., 1999; Zhu et al., 1998). Arf1 can potentially promote AP-1 binding to PI(4)P by recruiting/activating PI4Ks to increase the amount of available PI(4)P (Godi et al., 1999) or by generating a second docking site, presumably a protein (Zhu et al., 1999; Zhu et al., 1998), that binds AP-1 synergistically with PI(4)P.

The first possibility is unlikely because there is currently no evidence to suggest that Arf1 regulates PI4KIIα. PI4KIIα association with the Golgi is resistant to brefeldin A, and the kinase activity of the resident type II PI4K (almost certainly PI4KIIα) in the Golgi and in immature secretory granules is not regulated by Arf1 (Godi et al., 1999; Panaretou and Tooze, 2002). We therefore favor the second possibility that PI4KIIα and Arf1 generate two independent signals; each is necessary, but individually insufficient, for stable AP-1 association with the TGN membranes.

The proposed two-component docking mechanism (PI4KIIα-generated docking lipids and Arf-generated docking proteins) is similar to those used to anchor many low affinity phosphoinositide binding modules tightly to membranes (McLaughlin et al., 2002; Yin and Janmey, 2003). In most cases, the initial docking mediated by the phosphoinositides increases the probability of binding to the truly specific binding site elsewhere in the protein and the membrane. The situation for AP-1 is likely to be considerably more complex, because AP-1 binds many other ligands, and these combinatorial and cooperative binding events may be necessary to anchor AP-1 firmly to the TGN. It is particularly intriguing that epsinR, an AP-1

accessory protein that is recruited to the TGN by Arf1, also binds PI(4)P (Hirst et al., 2003; Mills et al., 2003). Thus, the recruitment of epsinR and AP-1 to a common PI(4)P-rich membrane patch may synergistically increase their affinity for each other and for PI(4)P. In this regard, the dramatic effect of PI4KIIα RNAi on AP-1 association points to an apical role of PI(4)P in the generation of this cascade.

Phosphoinositide Compartmentalization to Specify Organelle Identity

Unexpectedly, the plasma membrane has very little PI(4)P. We and others had previously assumed that the bulk of PI(4)P synthesized by the Golgi is exported to the plasma membrane constitutively (Cremona and De Camilli, 2001), where it awaits to be converted to PIP2 on demand. Our result suggests that this is unlikely to be the case, at least in cells that are not specialized for regulated exocytosis (such as HeLa). Instead, PI(4)P generated in the Golgi and Golgi-derived carriers is probably converted to PIP2 either en route to the plasma membrane or immediately after it reaches the plasma membrane.

The compartmentalization of PI(4)P and PIP2 between the Golgi and the plasma membrane raises the possibility that they may specify the differential recruitment of closely related effector proteins (Munro, 2002). PI(4)P-dependent recruitment of AP-1 to the Golgi distinguishes it from PIP2/PIP3-dependent recruitment of AP-2 to the plasma membrane (Collins et al., 2002; Gaidarov and Keen, 1999; Rohde et al., 2002). Likewise, epsin1 is recruited to the plasma membrane via PIP2 (Ford et al., 2002), and epsinR may be recruited to the Golgi via PI(4)P (Hirst et al., 2003; Mills et al., 2003).

The concept of lipid-defined organelle identity originated from the finding that endosomes are enriched in PI(3)P (Gillooly et al., 2000), and gained momentum when it was

discovered that the plasma membrane is particularly enriched in PIP2. Our discovery that AP-1 recruitment to the TGN is dependent on PI(4)P and that the Golgi accounts for the largest PI(4)P pool in cells strongly supports the possibility that PI(4)P is the predominant Golgi membrane marker (Munro, 2002; Roth, 2004). We therefore propose that PI(4)P is another arm of the membrane lipid recognition system.

Our hypothesis is also supported by more recent studies. De Matteis and colleagues have shown that association of FAPPs with the TGN is dependent on PI(4)P and regulated by Arf1 (De Matteis and Godi, 2004; Godi et al., 2004). The FAPPs have an important role in the generation of transport carriers destined to the plasma membrane from the TGN. Their TGN localization is determined by a PH domain that binds PI(4)P and Arf1 simultaneously. In Chapter 4, I will show that GGAs TGN recruitment is also dually controlled by PI(4)P and Arf1.

Altogether, PI(4)P is the common regulator for TGN recruitment of AP-1, EpsinR, FAPPs and GGAs. It defines TGN lipid organelle identity by increasing the affinity of TGN membranes for peripheral membrane proteins that are important for membrane traffic.

Materials and Methods

Cell Culture, Plasmid Transfections, and Adenovirus Infections

Cells were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected with lipofectAMINE (Invitrogen), or transfected with siRNA using oligofectAMINE (Invitrogen).

Antibodies, Constructs, and Reagents

The rabbit polyclonal anti-PI4KIIα (Wei et al., 2002), anti-PI4KIIβ (Wei et al., 2002), monoclonal anti-VSVG, and anti-HA antibodies (Lin et al., 1998; Lin et al., 1997) were described previously. Antibodies from commercial sources are as follows: rabbit anti-PI4KIIIβ (Upstate Biotechnology), goat anti-PIP5KIα (Santa Cruz Biotechnology), sheep anti-TGN46 (Serotec), monoclonal anti-PI(4)P and anti-PIP2 (Assay Designs, Inc.), monoclonal anti-γ-adaptin (clone 100/3; Sigma), monoclonal anti-β-COP (clone M3A5; Sigma), anti-MPR300 (Affinity Bioreagents, Inc), and anti-actin (Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch Labs, Inc., Amersham Life Sciences, or Santa Cruz Biotechnology.

GFP-OSBP-PH construct (Levine and Munro, 2002) was a gift of S. Munro, and pECFP-Golgi (CFP-β-GT) was from Clontech Labs, Inc.

Most other non-tissue culture reagents were from Sigma, except as noted in the text.

PI4KIIα RNA Interference

The siRNA sequence targeting human PI4KIIα (GenBank accession number NM_18425) spans nucleotides 888–908 and is specific for hPI4KIIα based on BLAST search (NCBI database). A siRNA sequence corresponding to nucleotides 695–715 of the firefly luciferase (U31240) was used as a negative control. siRNAs were synthesized by the Center for Biomedical Inventions (U. of Texas Southwestern Medical Center at Dallas), and annealed according to the protocol recommended by Dharmacon Research, Inc.

HeLa cells were plated in 6-well plates at 20%–30% confluence for 24 hr and transfected with 10 μl of 20 μM siRNA and 3 μl of oligofectAMINE in 1 ml of Opti-MEM. After 5 hr, cells were washed and cultured in DMEM containing FBS. They were either left alone, transfected

with a cDNA (such as GFP-OSBP-PH), or infected with virus and used 72 hr after the initial siRNA treatment.

Immunofluorescence Microscopy

siRNA-treated cells were trypsinized after transfection, and reseeded on glass coverslips. In most cases, cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 on ice, and labeled with antibodies in blocking buffer (1% BSA, 3% donkey serum in PBS). In some cases, cells were fixed as above and "cracked open" by freeze-thawing without using detergents (Tran et al., 1999).

Cells were examined by a Zeiss 510 Laser Scanning Confocal Microscope using a 63 × 1.3 NA PlanApo objective. The Golgi is defined as the perinuclear region that is stained by anti-TGN46. This region of interest was selected, and the intensity of the other markers in this region is considered as Golgi-associated protein fluorescence. The time of image acquisition, the image gain, and enhancement were optimally adjusted at the outset and kept constant for all samples. In most cases, images were collected near the middle of the *z* axis. Captured images were analyzed using Metamorph Image software. Pixel intensity was used to quantitate fluorescence in the region of interest. Fluorescence intensity of Golgi AP-1 was expressed in arbitrary units after subtracting background cytosolic AP-1; VSVG and PI(4)P fluorescence in the Golgi were expressed as percentage of total fluorescence in the cell.

Phospholipid Analyses

Cells were labeled for 4 hr with 40 μCi/ml ^{32}P-PO_4 (NEN) in phosphate-free DMEM. Lipids were extracted with $CHCl_3$:methanol:HCl (volume ratio 5:10:4), resolved by thin layer chromatography (TLC) (Yamamoto et al., 2001), and detected using a Phosphorimager.

***In Vitro* AP-1 Membrane Binding Assay**

HeLa cells were homogenized and centrifuged at 100,000 × g (Wei et al., 2002). The microsome pellet was resuspended and incubated for 10 min with ice-cold high salt alkaline solution (1 M NaCl, 0.1 M sodium carbonate [pH 10.0]) to remove membrane-bound AP-1. The stripped membranes were collected by centrifugation at 20,000 × g for 15 min, washed once, and resuspended in binding assay buffer (25 mM HEPES-KOH [pH 7.0], 250 mM sucrose, 125 mM potassium acetate, 5 mM magnesium acetate supplemented with 1 mM dithiothreitol [DTT]) at 1 mg protein/ml.

25 μl of the stripped membranes was incubated with 10 μl of 1 mg/ml of antibodies at 25°C for 15 min. 170 μl of HeLa cytosol (Traub et al., 1993) was added, and the final incubation mixture contained 100 μM GTPγS, 1 mM ATP, 8 mM creatine phosphate, 80 μg/ml creatine kinase, and 1 mM DTT. After incubation at 37°C for 15 min, samples were diluted with 400 μl binding assay buffer without sucrose, centrifuged at 20,000 × g for 15 min at 4°C, washed once, and analyzed by SDS-PAGE and Western blotting.

Protein-Lipid Overlay Assay

Bovine AP-1 was purified from bovine brain clathrin-coated vesicles by gel filtration followed by hydroxyapaptite chromatography as described previously (Rapoport et al., 1998). AP-1 binding to phospholipids was performed at room temperature using PIP-Strip and PIP-Array (Echelon Biosciences, Inc) following the manufacturer's protocol. Bound AP-1 was detected with anti-γ-adaptin and HRP-conjugated anti-mouse IgG.

Rescue of AP-1 Binding in Intact and Semi-Intact Cells by Exogenous Phospholipids

Intracellular Delivery of PPIs into Intact Cells

We used the Echelon ShuttlePIP kit (Echelon Biosciences, Inc.) (Ozaki et al., 2000). Cells were washed with serum free DMEM twice, followed by incubation at 37°C for 30 min in serum-free DMEM with ShuttlePIP components. 10–20 μM diC16 PI(4)P and diC16 PIP2 were delivered intracellularly (shuttled) via Echelon's polyamine carriers 3 and 2, respectively.

Addition to Semi-Intact Cells

AP-1 recruitment in semi-intact cells was performed as described by Zhu et al. (1998). siRNA-treated cells were permeablized with 20 μg/ml digitonin on ice for 10 min in 25 mM HEPES-KOH (pH 7.2), 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, and 1 mg/ml D-glucose. They were further incubated at 37°C for 5 min in permeabilization buffer without digitonin to strip off endogenous AP-1.

The permeabilized cells were incubated with 20 μM sonicated PI(4)P or PIP2 in 25 mM HEPES-KOH (pH 7.2) and 1 mg/ml D-glucose for 15 min at room temperature. Four volumes

of HeLa cytosol were added together with 100 μM GTPγS and an ATP-regeneration system. After 15 min incubation at 4°C, the coverslips were washed twice with PBS and cells were fixed with methanol for immunofluorescence analysis.

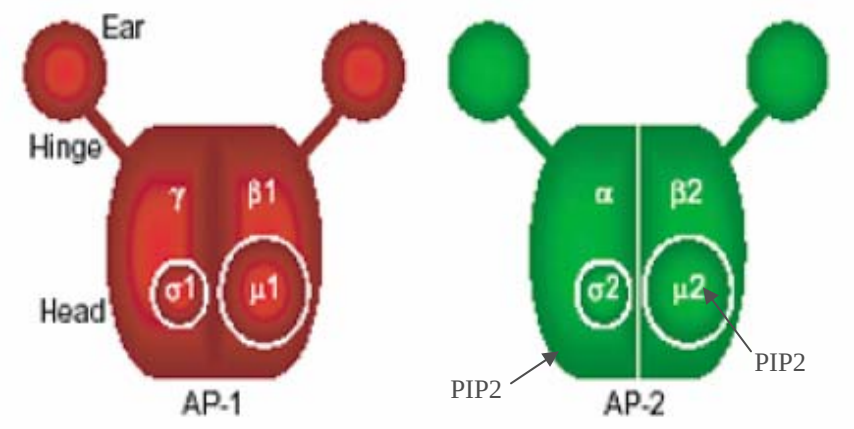

Ear
Hinge
γ
β1
σ1
μ1
Head
AP-1
α
β2
σ2
μ2
PIP2
PIP2
AP-2

Figure 5. Schematic diagram of AP-1 and AP-2 (adapted from *Robinson and Bonifacino, 2001*). AP-1 has two large subunits (γ- and β1-adaptins) and two smaller subunits (μ1 and σ1). The large adaptins have an N-terminal head domain, a variable hinge region and a C-terminal ear domain. The ears and hinges are referred to as "appendages" and the entire complex without the appendages are referred to as "cores". AP-2 has a similar subunit organization. Arrows point to PIP2 binding sites determined by crystallography (Collins et al, 2002).

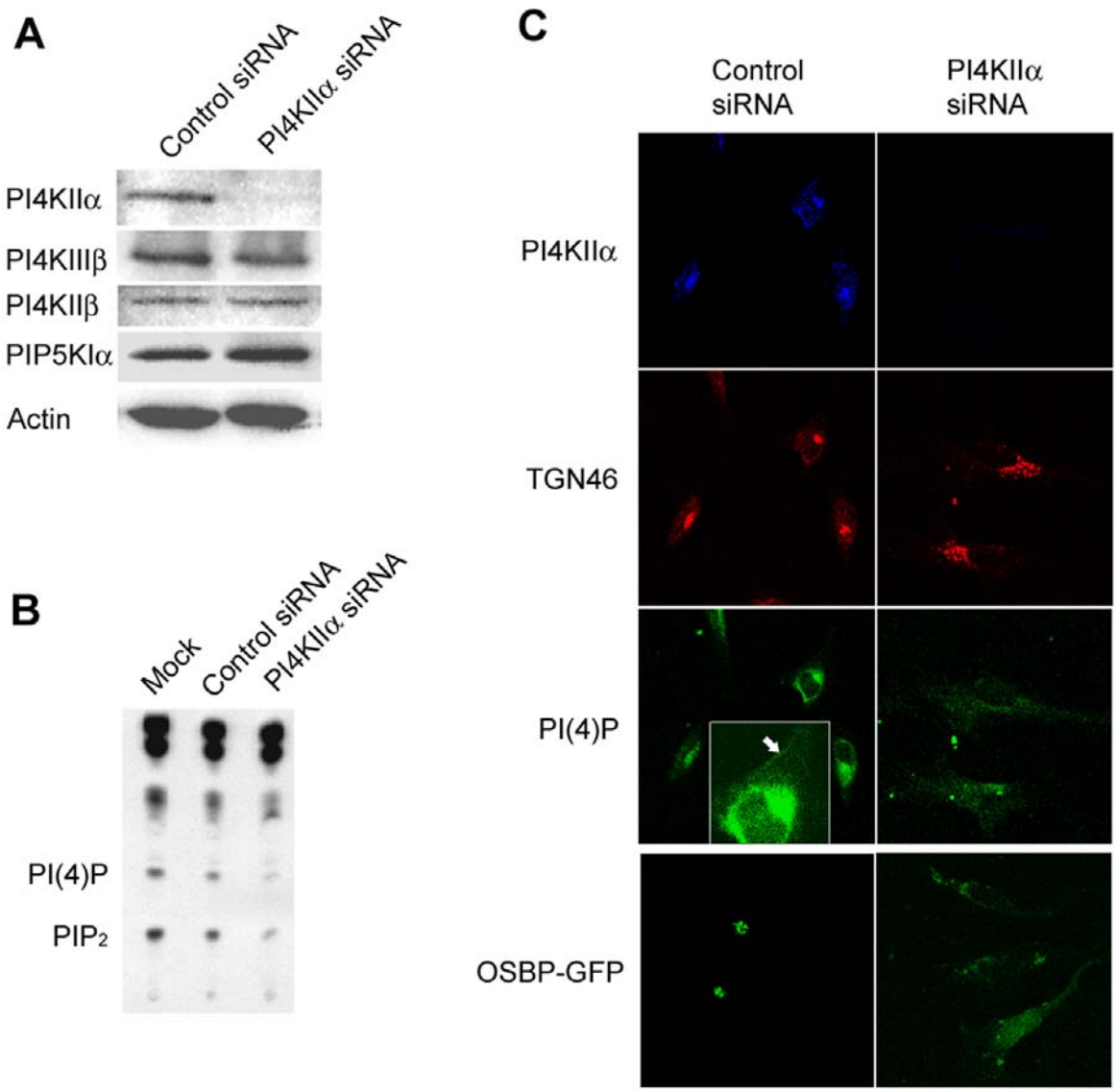

A
Control siRNA
PI4KIIα siRNA
PI4KIIα
PI4KIIIβ
PI4KIIβ
PIP5KIα
Actin
B
Mock
Control siRNA
PI4KIIα siRNA
PI(4)P
PIP2
C
Control siRNA
PI4KIIα siRNA
PI4KIIα
TGN46
PI(4)P
OSBP-GFP

Figure 6. Effects of PI4KIIα RNAi on Golgi morphology and phosphoinositide content. HeLa cells were harvested or fixed 72 hr after the start of siRNA transfection. (A) Western blotting of PI4KIIα, other phosphoinositide kinases, and actin. 10 μg of total cell proteins was loaded per lane. (B) ^{32}P incorporation into lipids, analyzed by TLC and autoradiography. Mock refers to treatment with oligofectAMINE in the absence of added siRNA. Data is representative of more than 3 independent experiments. (C) Immunolocalization of PI(4)P. Cells were fixed with formaldehyde and cracked open by freeze/thawing. Top three rows, control and PI4KIIα RNAi cells were triple stained. Inset shows an enlarged image of a control cell which was image-enhanced to highlight the small amount of anti-PI(4)P staining at the plasma membrane (indicated by arrow). Bottom row shows control and PI4KIIα RNAi cells that were transfected with OSBP-PH-GFP cDNA 18 hr prior to fixation.

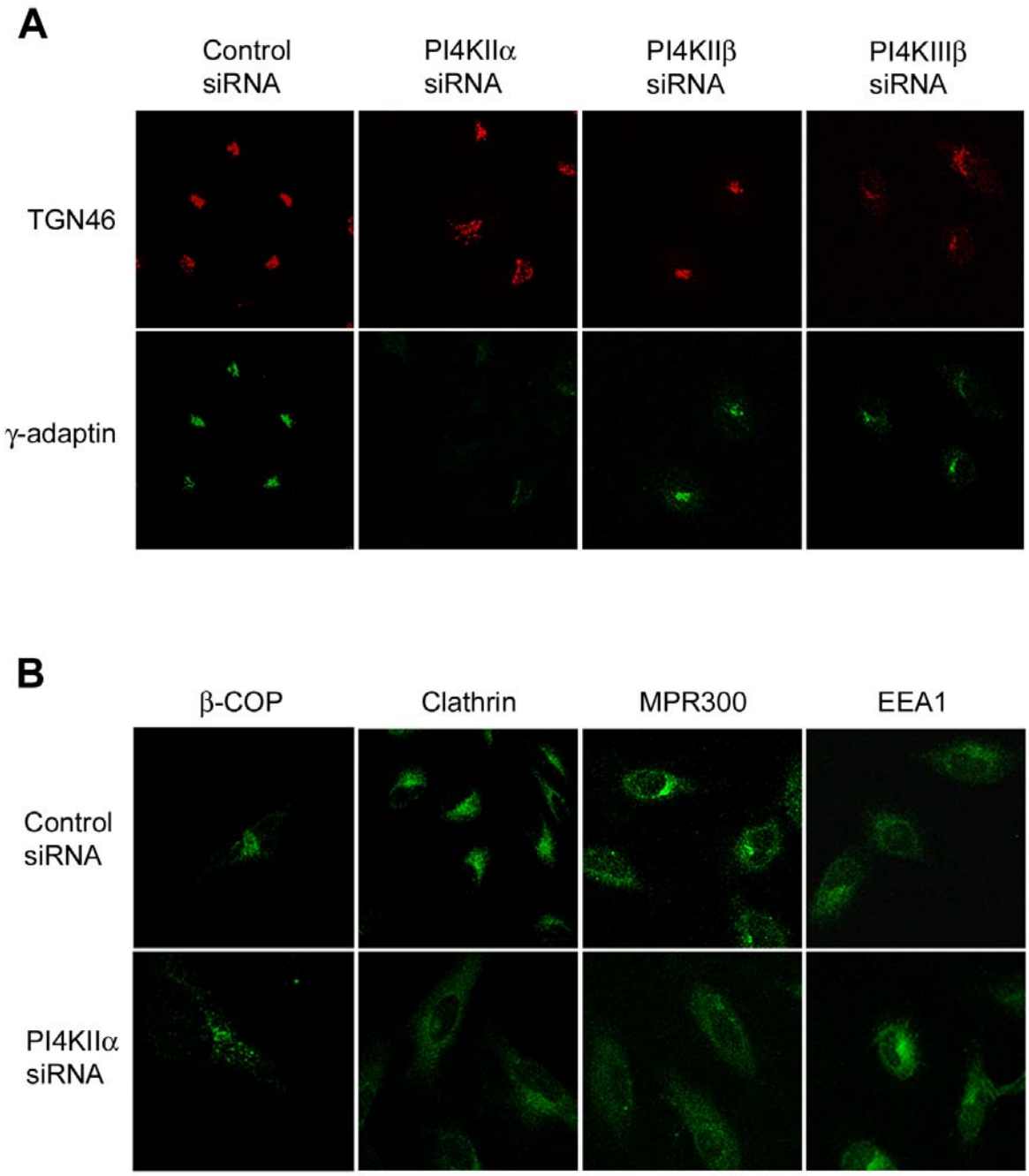
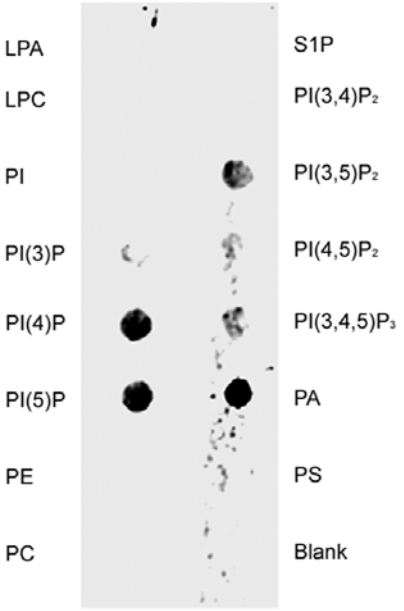

A
Control siRNA
PI4KIIα siRNA
PI4KIIβ siRNA
PI4KIIIβ siRNA
TGN46
γ-adaptin
B
β-COP
Clathrin
MPR300
EEA1
Control siRNA
PI4KIIα siRNA

Figure 7. Effects of PI4KIIα RNAi on AP-1 recruitment. (A) Comparing the effects of knocking down each of the three Golgi-associated PI4Ks. Cells were triple labeled, and TGN46 and γ-adaptin images in the same field are shown. Isoform specific anti-PI4K antibodies confirmed that there was knockdown of each kinase. (B) Control and PI4KIIα RNAi cells were stained with the antibodies to the proteins indicated. Although not shown, PI4KIIα knockdown and Golgi localization of these markers were confirmed by anti-PI4KIIα and anti-TGN46 staining in all cases. Images were collected at optical sections at the middle of the cell.

A


LPA
LPC
PI
PI(3)P
PI(4)P
PI(5)P
PE
PC
S1P
PI(3,4)P2
PI(3,5)P2
PI(4,5)P2
PI(3,4,5)P3
PA
PS
Blank

B

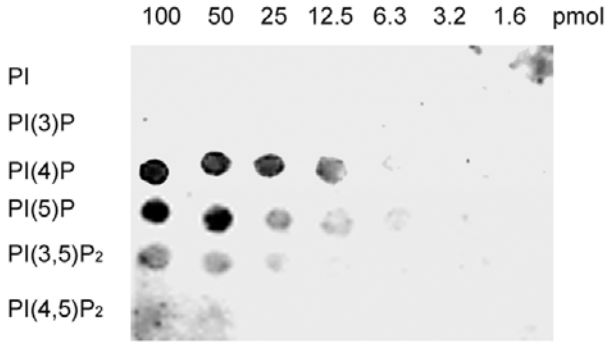

100 50 25 12.5 6.3 3.2 1.6 pmol
PI
PI(3)P
PI(4)P
PI(5)P
PI(3,5)P2
PI(4,5)P2

Figure 8. AP-1 binding in a protein:lipid overlay assay. Lipids dotted strips (Echelon Biosciences) were incubated with purified AP-1, and bound γ-adaptin was detected with antibody. (A) The PIP-Strip was dotted with 100 pmol of each of the lipids indicated. (B) The PIP-Array was dotted with different amounts of selected lipids. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; S1P, sphinogosine 1-phosphate; PA, phosphatidic acid.

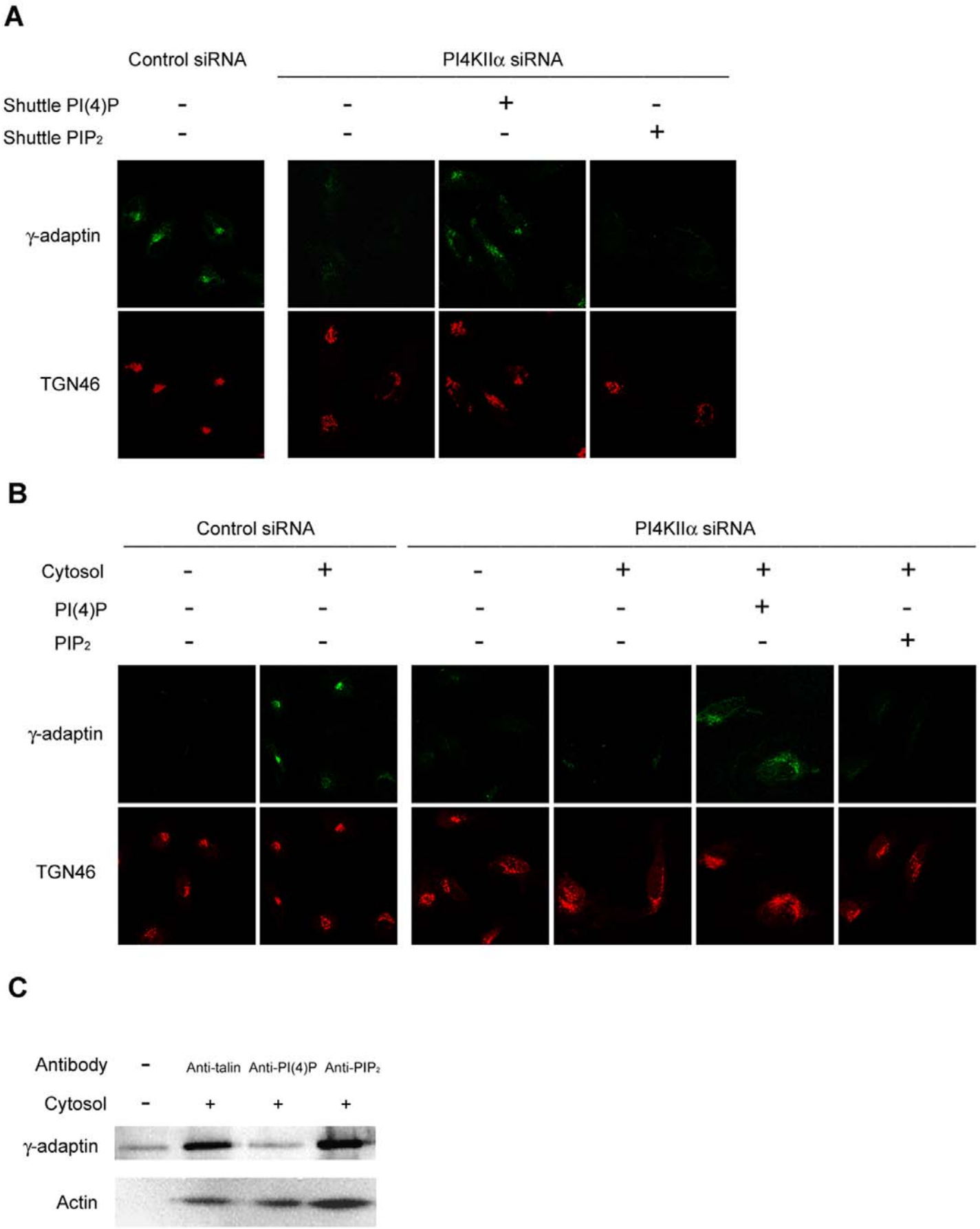

A
Control siRNA
PI4KIIα siRNA
Shuttle PI(4)P - - + -
Shuttle PIP2 - - - +
γ-adaptin
TGN46
B
Control siRNA
PI4KIIα siRNA
Cytosol - + - + + +
PI(4)P - - - - + -
PIP2 - - - - - +
γ-adaptin
TGN46
C
Antibody - Anti-talin Anti-PI(4)P Anti-PIP2
Cytosol - + + +
γ-adaptin
Actin

Figure 9. PI(4)P is required for AP-1 association with the Golgi. (A) In vivo rescue of γ-adaptin (AP-1) binding by shuttle PI(4)P. PI4KIIα RNAi cells were incubated with polyamine shuttle carriers in the presence or absence of 20 μM diC16-PI(4)P or diC16-PIP2 for 30 min at 37°C. Cells were fixed, permeabilized with Triton X-100, and triple stained with anti-γ-adaptin, anti-TGN46, and anti-PI4KIIα. γ-adaptin and TGN46 images are shown. (B) PI(4)P addback restores AP-1 binding to the Golgi of semi-intact cells. Control and PI4KIIα RNAi HeLa cells that were permeabilized with digitonin and salt stripped were incubated with concentrated HeLa cytosol in the presence or absence of 20 μM PI(4)P or PIP2 for 15 min at room temperature. Cells were triple labeled with anti-PI4KIIα (not shown), anti-γ-adaptin, and anti-TGN46. (C) Anti-PI(4)P blocks AP-1 binding to normal membranes in vitro. HeLa microsome membranes (not treated with siRNA) that were first stripped with high salt to remove bound AP-1 were treated with anti-PI(4)P, anti-PIP2, or anti-talin. They were then exposed to HeLa cytosol. γ-adaptin and actin (as a control) were detected by Western blotting.

Table 2. Effects of PI4KIIa RNAi and Shuttle PPIs on Intact Cells

	% intensity in TGN *		TGN γ-adaptin	
	PI(4)P	TGN46	Intensity (A.U.)#	% cells with TGN staining&
Control	46.5±1.7	82.1±1.2	12.1±1.4	89
PI4KIIα RNAi				
Carrier	16.7±1.4	76.6±1.6	3.6±0.5	29
Carrier+PIP	ND	85.9±1.8	7.0±0.5	79
Carrier+PIP2	ND	92.6±0.8	4.2±0.9	6

*Values are mean ± SEM of 8-10 randomly chosen cells per condition.
Values are mean ± SEM of 8-10 cells with TGN γ-adaptin staining.
& Approximately 50 randomly chosen cells were scored per condition.
A.U., arbitrary units

Table 3. Effects of PI(4)P and PIP2 on AP-1 Recruitment to the TGN of permeabilized cells

	TGN γ-adaptin Intensity#	%cells with TGN γ-adaptin staining&
Control w/o cytosol	5.2±0.2	7
Control with cytosol	13.0±0.7	57
PI4KIIα RNAi		
w/o cytosol	3.0±0.3	5
with cytosol	3.4±0.2	4
with cytosol+PIP	6.9±0.2	79
with cytosol+PIP2	3.2±0.3	6

[#] Values are mean ± SEM of 10 cells. &15- 30 randomly chosen cells were scored per condition.

CHAPTER 3

Characterization of AP-1 Binding to PI(4)P and Arf1

Introduction

The heterotetrameric AP complexes are assemblies of two related but distinct large chains, a medium chain, and a small chain. Mammalian cells have four such heterotetrameric APs, designated AP-1 through AP-4, each of which works in a particular traffic route: AP-1 (γ, β1, μ1, and σ1) (Figure 5), AP-3 (δ, β3, μ3, and σ3), and AP-4 (ε, β4, μ4, and σ4) in the trans-Golgi network (TGN) and endosomes and AP-2 (α, β2, μ2, and σ2) (Figure 5) at the plasma membrane (Robinson, 2004; Robinson and Bonifacino, 2001). With the exception of AP-2, all are recruited to TGN in an Arf1-GTP dependent manner.

APs have a "core" and two "appendages". The core comprises the two 70-kDa N-terminal domains ("trunks") of the large chains, the 50-kDa μ chain, and the 20-kDa σ chain. The appendages are C-terminal domains of each of the large chains, connected to the rest of the structure by extended hinge segments (Figure 5). The structure of the AP-2 core in complex with IP6 (a PIP2 mimetic) has been determined by Owen and coworkers (Collins et al., 2002). They have suggested that their core structure represents an inactive state, and that activation requires an outward displacement of μ2C to expose the contact site.

The structure of the AP-2 core reveals a site near the N terminus of the α chain that could determine the interaction of AP complexes with phosphoinositides. The AP-2/IP6 creates a lattice contact by bridging one corner of the α chain and a positively charged patch on the μ2 chain of another complex. Intracellular localization experiments with mutated overexpressed α chain suggest that the α-chain site participates in a functional interaction with the headgroup of

PI(3,4,5)P3, a component of the plasma membrane (Gaidarov et al., 1996). Mutational experiments based on the structure also implicate the μ2 basic patch in binding to liposomes bearing PI(4,5)P2 and to the plasma membrane (Rohde et al., 2002), but μ1 in AP-1 lacks this concentration of positive charge.

Harrison and his colleagues have determined the structure of the AP-1 in the absence of PI(4)P or IP6. Its organization is similar to that of the core of AP-2, although their crystal contacts are completely different. The corner of the AP-1 γ chain that corresponds to the AP-2 α chain's IP6-binding site differs noticeably from that of its α counterpart (Figure 10).

Here we show that directed mutations of selected residues at this corner interfere with AP-1 recruitment to the TGN and decrease liposome binding of AP-1 cores *in vitro*, but without affecting the interaction of AP-1 with Arf1 complexed to GTPγS. The data suggest that this site helps to determine the association of AP-1 with Arf1-bearing membrane compartments by interacting with negatively charged lipid headgroups like that of PI(4)P.

Results

Potential Phosphoinositide-Binding Sites

We have not been able to obtain AP1:PI(4)P complexes by soaking the AP-1 core crystals with any of a variety of potential phosphoinositide or inositol phosphate ligands or by cocrystallization. However, we can deduce aspects of a potential interaction with PI(4)P or PI(4,5)P2 by reference to the IP6 site in the AP-2 crystals, where the ligand required for crystallization lies at a molecular contact (Collins et al., 2002). One face of that contact is a notch created by helices 2 and 3 of the α subunit and by the loop connecting them. The other face is a positively charged patch on μ2C not conserved in μ1. Residues in the α subunit that

contact IP6 include αArg-11, αLys-43, αTyr-53, αLys-57, αTyr-58, and αLys-61, as well as the backbone amide of αGly-12. A triple mutation of Lys-55, -56, and -57 to Gln prevents recruitment to plasma membrane-coated pits and overexpression of the mutant inhibits plasma membrane localization of AP-2, presumably by displacing the endogenous wild-type α chain (Gaidarov and Keen, 1999). Thus, there is evidence that the α-chain site is indeed important for membrane association. Of the residues that contact IP6 in the AP-2 crystals, only αLys-57 and -61 are conserved in the γ subunit as γArg-48 and γLys-52, and the overall charge density in the region is lower, consistent with preference for a monophosphoinositide with one rather than two or three phosphates.

Inspection of the corner between helices 2 and 3 suggested to us that γTyr-45, γArg-48, and γLys-52 would be good candidates for phosphoinositide head-group contacts (Figure 11). We mutated each of these residues to Ala and asked whether the changes affect TGN targeting of the modified AP-1 complex. We also included Arg-6 in this series of mutations to examine a possible role for the N-terminal segment.

Effects of Mutations at Potential Phosphoinositide-Binding Site on TGN Targeting

To study the effects of these mutations, we transfected HeLa cells with constructs encoding wild-type and mutant AP-1 γ chain tagged with enhanced GFP (EGFP). We confirmed, as reported (Puertollano et al., 2003), that most of the EGFP-tagged wild-type γ concentrates in the perinuclear area, with the remainder in more peripheral sites (Figure 12 *Top*). We also confirmed that reduction of PI4K IIα by RNA interference prevented this perinuclear localization (data not shown), just as it blocked similar localization of endogenous γ (incorporated into AP-1), detected by immunofluorescence (Wang et al., 2003). The targeting of the recombinant tagged

γ chain is thus just like that of the endogenous protein. The images in Figure 11 show that mutation of any one of residues 45, 48, or 52 to Ala in γ-adaptin prevents normal perinuclear targeting, but that mutation of Arg 6 to Glu, which projects away from the proposed site for PI(4)P, has no effect. The integrity of the TGN and Golgi compartments was maintained in these experiments, because expression of the mutant proteins did not affect targeting of the TGN marker TGN46 (Figure 12) or β-COPI (a marker for the Golgi compartments; data not shown). Expression in insect cells of any of these mutated γ chains together with the other three chains of AP-1 yielded assembled complexes with native-like properties (Figure 13), showing that the mutant γ subunits incorporate normally into AP-1, and that failure of TGN targeting is not a consequence of reduced complex formation.

Effects of Mutations at Potential Phosphoinositide-Binding Site on Liposome Binding

Because recruitment of AP-1 to Golgi membranes depends not only on active PI4KIIα but also on the small GTPase Arf1 in its GTP-bound state (Stamnes and Rothman, 1993), we examined the effects of two of the γ chain mutations on the association of recombinant AP-1 cores with liposomes containing Arf1 or PI(4)P. As expected, myristoylated Arf1, activated by binding of GTPγS, enhanced association of wild-type AP-1 core with liposomes containing PS (Zhu et al., 1998). AP-1 cores containing γ subunits with alanine substituted for Y45 or R48 responded similarly (Figure 14). We conclude that these mutations do not affect a critical Arf1-binding interface. Incorporation of PI(4)P into the PS-containing liposomes also enhanced AP-1 association, establishing that AP-1 interacts with PI(4)P.

The R48A mutation eliminated the PI(4)P enhancement of AP-1 recruitment to liposomes, but it did not impair background interaction with PS (Figure 14). Thus, the loss of a positive

charge alters specificity but does not destroy affinity for negatively charged lipids generally. We propose that R48 is important for Golgi targeting because of its interaction with PI(4)P, and that in the absence of this contact, Arf1 does not suffice. It is remarkable that despite the extensive network of parallel interactions in which the nearly 300-kDa AP-1 complex participates, a single point mutation at a lipid–head-group interaction site is sufficient to disrupt correct intracellular localization.

The Y45A mutation also disrupts localization, but it does not affect PI(4)P or Arf1 enhancement in our liposome-binding assay (Figure 14). We cannot resolve here the difference in apparent behavior. It is possible that there are subtle changes in affinity that are not detected by the *in vitro* assay. In some of our intracellular localization experiments, it appeared that this mutation might have a weaker effect than R48A, but we have not yet established a firm correlation.

Discussion

In its requirement for both a small GTPase and a phosphoinositide, AP-1 resembles a number of other proteins that are recruited to specific intracellular membrane compartments. Examples include OSBP and FAPP1, which recognize the presence of Arf1 and PI(4)P in the TGN (Godi et al., 2004; Levine and Munro, 2002). However, the structural basis of stereochemical specificity for a particular headgroup geometry has been difficult to demonstrate.

Metabolism of phosphoinositides is under tight temporal and spatial control. One form of membrane identity seems to be provided by the relative enrichment of specific phosphoinositides in a given membrane (Munro, 2004; Roth, 2004). The properties and structures of AP-1 and AP-2 cores suggest that adaptors, rather than the cargo they sort, are the critical detectors of

phosphoinositide content. Their relatively low affinity and modest specificity for particular phosphoinositides can be understood by recognizing that they are coincidence detectors. Thus, AP-1 responds to the combined presence of PI(4)P and Arf1, which draw it to locations where it can then interact with both cargo and clathrin. The structure described here is a step toward understanding the properties and intricacies of such networks.

Materials and Methods

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the QuickChange kit (Stratagene). PCRs were performed using an overlap extension by *Pfu* Turbo DNA polymerase with GFP-γ adaptin as templates. Mutations were confirmed by nucleotide sequencing analysis.

Expression and Purification of Proteins

cDNA-encoding residues 1–584 of rat β1, 1–423 of mouse μ1A, and 1–158 of mouse σ1A adaptins were each subcloned into pFastBac1 vectors; cDNA encoding residues 1–613 of mouse γ-adaptin was subcloned into a pFastBacHTb vector. Recombinant baculoviruses were generated with the Bac-to-Bac baculovirus expression system (BD Biosciences). The heterotetrameric complex of AP-1 core was expressed by coinfection of Hi5 cells with all four recombinant baculoviruses. The pure AP-1 cores were concentrated to between 30 and 40 μM in 20 mM Tris·HCl/100 mM NaCl/2 mM 2-mercaptoethanol/1 mM EDTA, by using a 50,000 M_r Ultra15 concentrator (Millipore) and samples were stored at 4°C. Myristoylated Arf1 was purified as described (Franco et al., 1995) after its generation in *Escherichia coli* by coexpression of Arf1 and yeast *N*-myristoyltransferase (Duronio et al., 1990).

Preparation of Vesicles and Lipid-Binding Assay

Combinations of dioleoyl phosphatidylcholine (PC), dipalmitoyl phosphatidylethanolamine (PE), brain phosphatidylserine (PS), and brain PI(4)P (Sigma) (0.5 mg final) were dissolved in a glass tube in 200 µl of chloroform/methanol (2/1). The solvents were removed with a stream of nitrogen followed by overnight exposure to vacuum. The lipids were resuspended by vortex mixing for 20 s at room temperature with 100 µl of Tris buffer (10 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM DTT) and the vesicles (5 mg/ml) were stored at 4°C and used within 1 week of preparation.

AP-1 cores (0.2 µM) containing the wild type or the γ-adaptin mutants Y45A or R48A were mixed with the vesicles (1 mg/ml) for 15 min at room temperature in a final volume of 75 µl (Tris buffer). The samples were then subjected to centrifugation for 10 min at 200,000 x *g* at 25°C (TL100, Beckman Coulter) and the supernatants were removed. The pellets were resuspended with 30 µl of Tris buffer and supernatants and pellets were subjected to SDS/PAGE, Coomassie blue staining, and densitometry. Myristoylated Arf1 (myrArf1) (8 µM dissolved in Tris buffer containing 1 mM $MgCl_2$ and 2 mM EDTA) was preloaded with GTPγS (40 µM) for 45 min at 37°C. Vesicles containing myrArf1/GTPγS were centrifuged, resuspended in Tris buffer supplemented with 1 mM $MgCl_2$, and used for AP-1 binding as described above.

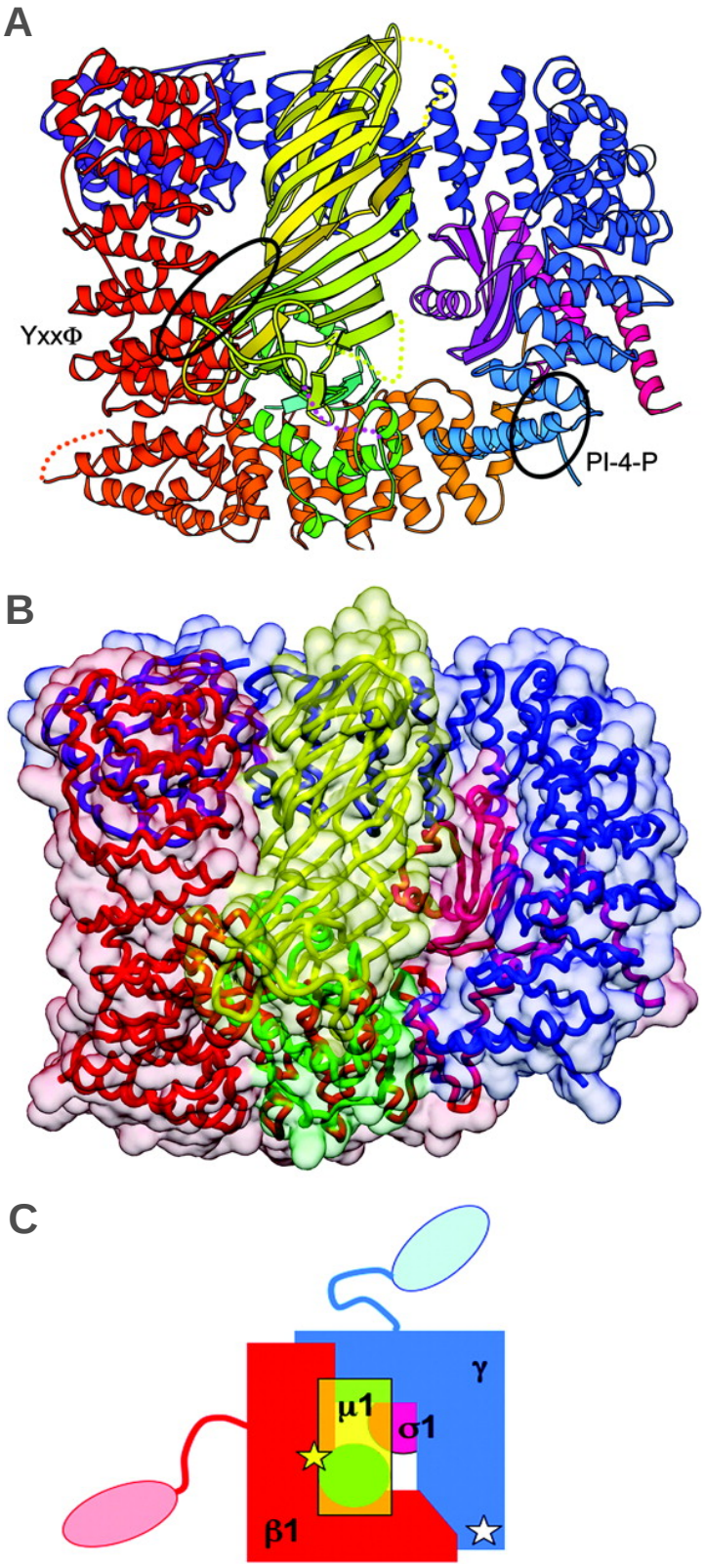

A
YxxΦ
PI-4-P
B
C
γ
μ1
σ1
β1

Figure 10. Structure of the AP-1 core. (A) Ribbon representation. The γ chain is blue; β1, red; μ1N, green; μ1C, yellow–orange; and σ1, magenta. Disordered segments are dotted lines of appropriate color. Proposed PI(4)P-binding site on γ and the Yxxϕ site on μ1 are shown by black ellipses. (B) Molecular surface representation. Colors as in A. (C) Diagram of AP-1. The core comprises the large-chain trunks (γ, blue; β1, red), the medium chain (μ1N, yellow–green; μ1C, yellow–orange), and the small chain (σ1, magenta). The appendages of the large chains are linked to the trunks by extended flexible connectors. Yellow star indicates binding site for tyrosine-based sorting signal; white star stands for a proposed binding site for inositol-phosphate headgroup.

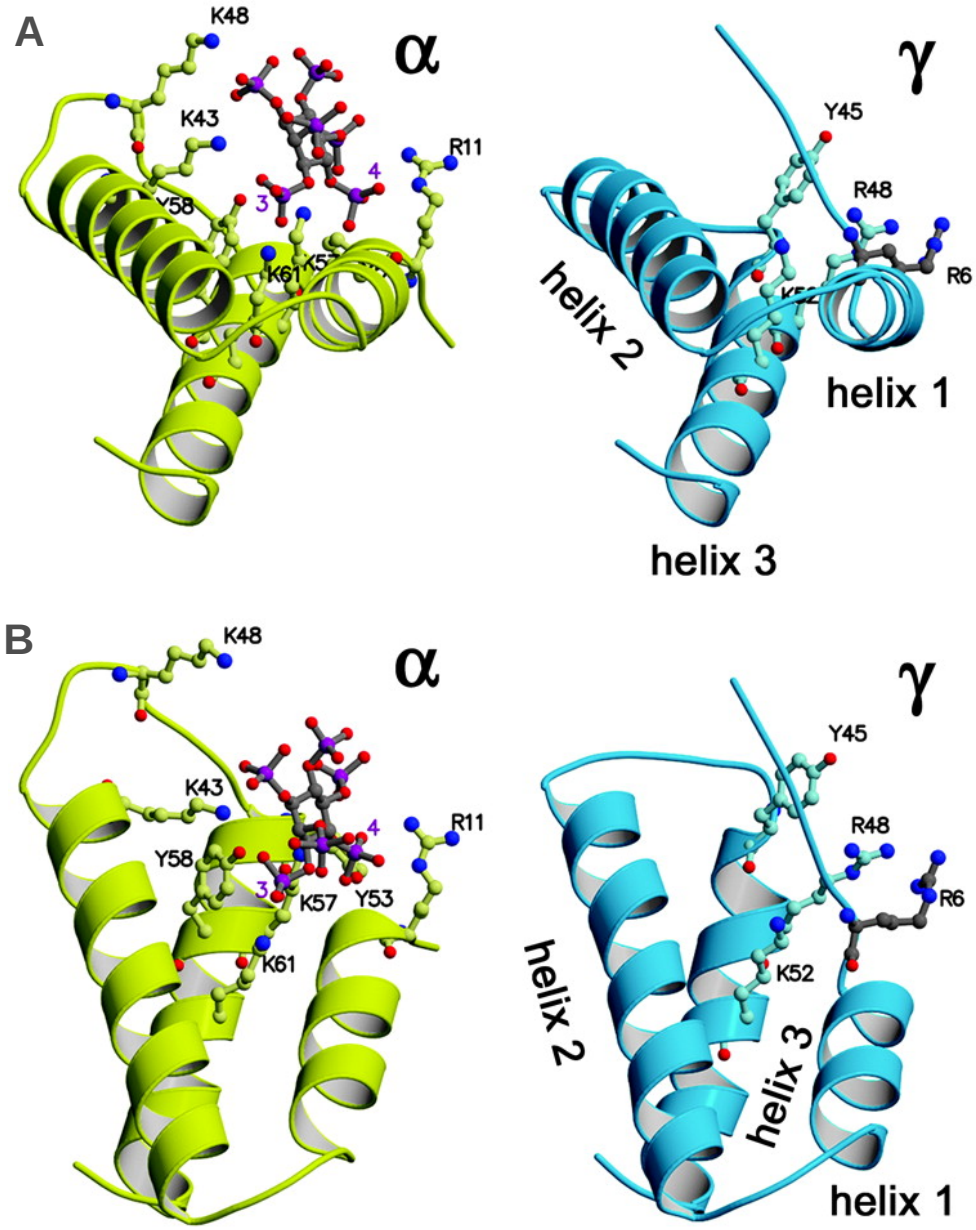

A
α
γ
K48
K43
R11
Y58
K61
Y45
R48
R6
helix 2
helix 1
helix 3
B
α
γ
K48
K43
R11
Y58
K57
Y53
K61
Y45
R48
R6
K52
helix 2
helix 3
helix 1
C
helix 1
helix 2
helix 3
γ MPAP-----IR-LRELIRTIRTARTQAEERE MIQKE
α MPAVSKGDGMRGLAVFISDIRNCKSKEAEIKRINKE
γ CAAIRSSFREE---DNTYRCRNVAKLLYMHMLGYP
α LANIRSKFKGDKALDGYSKKKYVCKLLFIFLLGHD

Figure 11. Comparison of the helix 2–helix 3 corner in the α chain (*Left*) of AP-2 and the γ chain (*Right*) of AP-1. (A) View as if looking from the left in Figure. 10A toward the corner enclosed in the oval; the top of the present figure corresponds to the bottom of Figure. 10. (B) The same as A, but rotated by 90° as shown. The α-adaptin of AP-2 is in yellow; the γ-adaptin of AP-1 is in cyan. The IP6 molecule and residues involved in binding phosphoinositides are in ball-and-stick representation (nitrogen, blue; oxygen, red; phosphorus, magenta; carbon, pale yellow in AP-2, pale cyan and gray in AP-1, and gray in IP6). (C) Amino acid sequences in this region and their secondary structures. The secondary-structure symbols are colored to match the ribbon diagram. Residues involved in phosphoinositide binding are in magenta (γ chain) and red (α chain); the residue R6 of γ chain is in green.

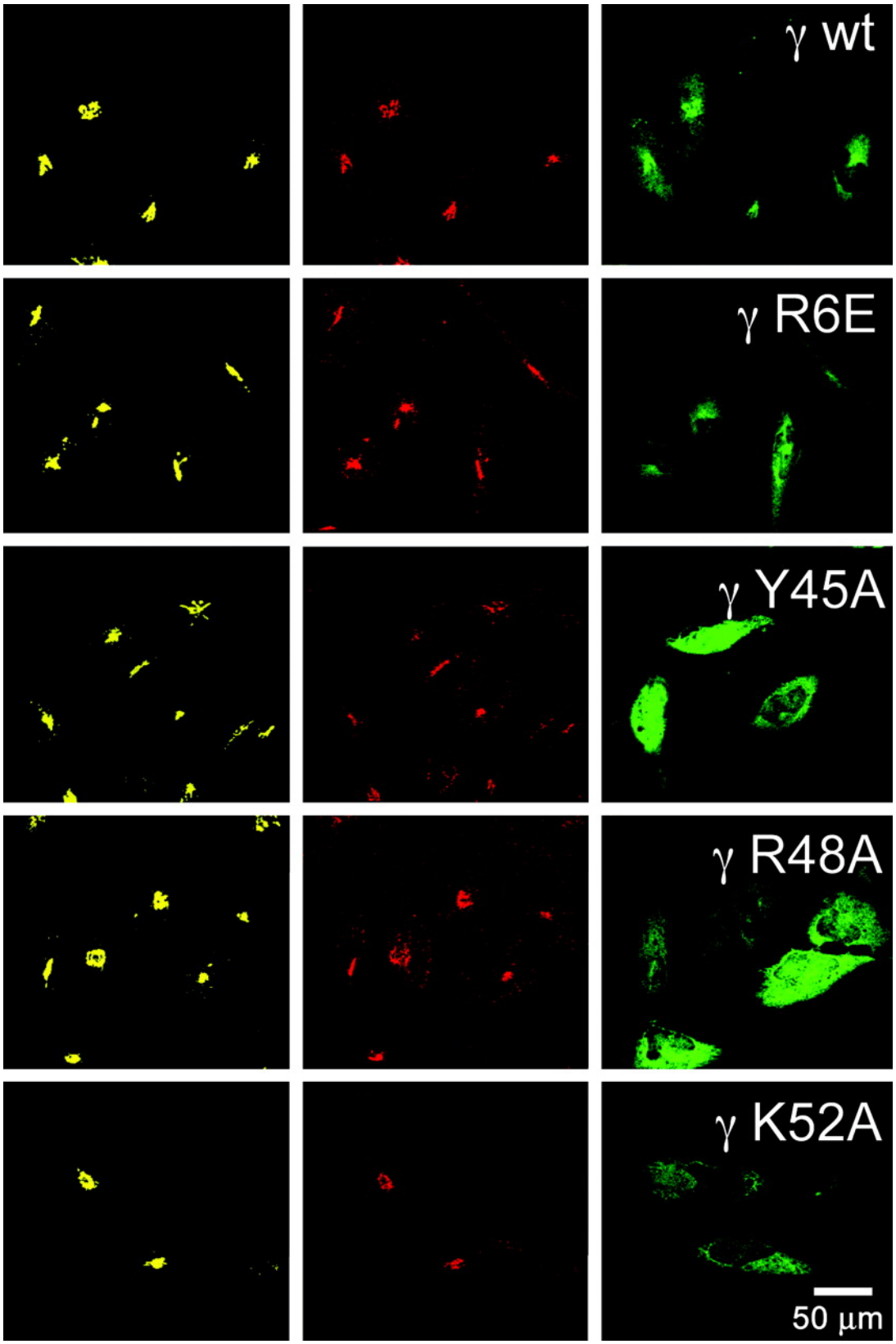

γ wt
γ R6E
γ Y45A
γ R48A
γ K52A
50 μm

Figure 12. Effects of mutations in γ chain on the TGN targeting of AP-1. HeLa cells transiently expressing wild-type or mutant (R6E, Y45A, R48A, and K52A) mouse γ-adaptin fused to enhanced GFP (green, *Right*) were fixed and fluorescently labeled with a sheep polyclonal antibody to TGN46 (yellow, *Left*) or a mouse monoclonal antibody 100/3 to human (but not mouse) γ -adaptin (endogenous γ; red, *Center*).

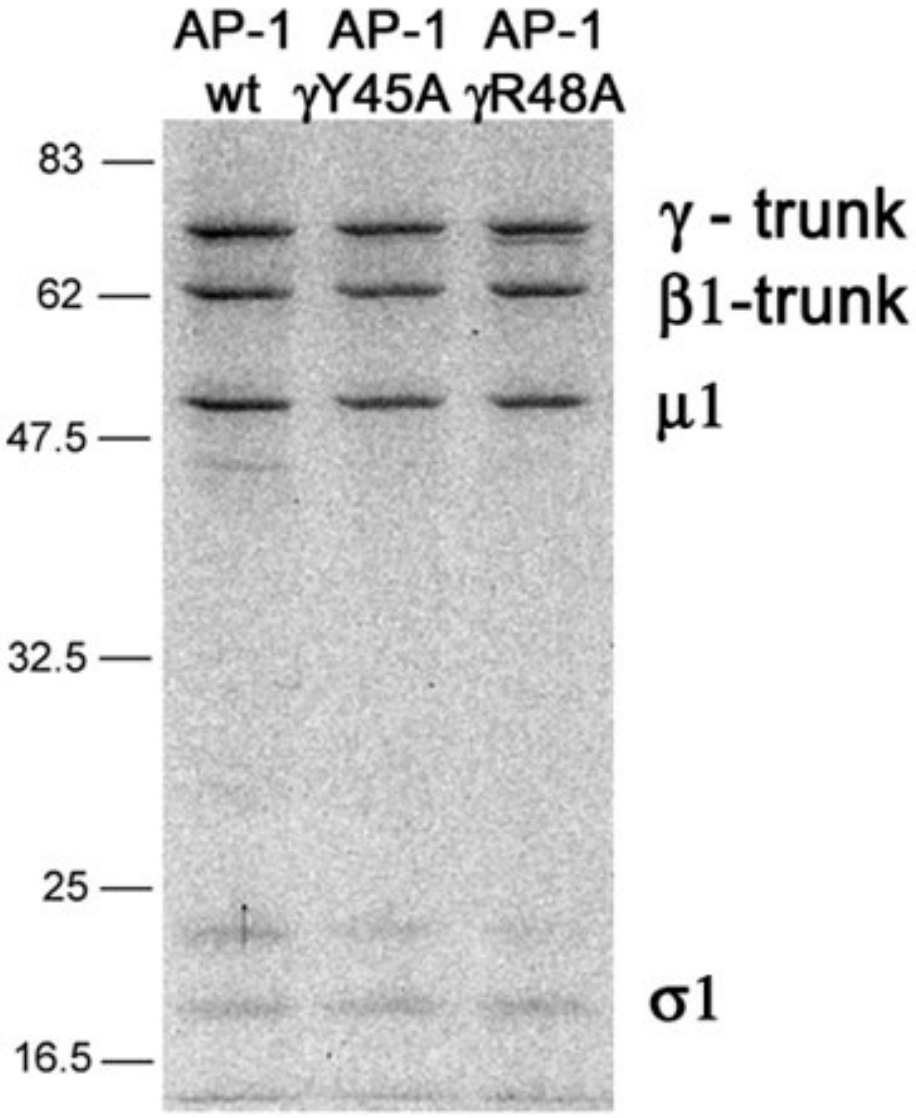

AP-1 wt
AP-1 γY45A
AP-1 γR48A
83
62
47.5
32.5
25
16.5
γ - trunk
β1-trunk
μ1
σ1

Figure 13. Subunit composition of recombinant AP-1 cores. Representative SDS/PAGE analysis of AP-1 cores containing wild-type and mutant γ -adaptins used in the lipid-binding assays. Cores were expressed in insect cells and purified as described. The samples were stained with Coomassie blue and the identity of the bands corresponding to the μ 1 and σ 1 chains confirmed by immunoblot analysis.

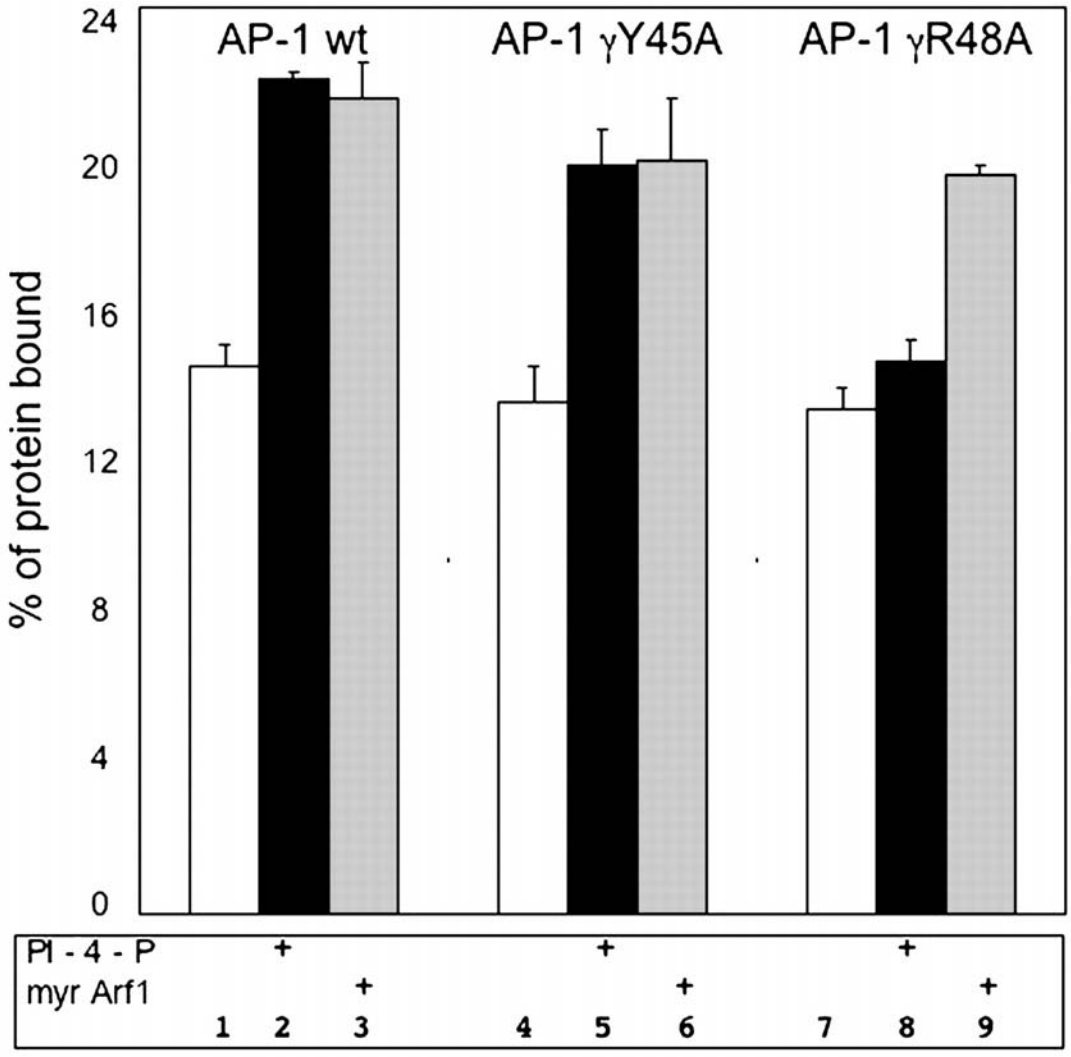

AP-1 wt
AP-1 γY45A
AP-1 γR48A
% of protein bound
24
20
16
12
8
4
0
PI - 4 - P
myr Arf1
1 2 3 4 5 6 7 8 9

Figure 14. Effects of mutations in γ chain on recruitment of AP-1 cores to liposomes. AP-1 cores (0.2 μM) containing either wild-type or mutant (Y45A or R48A) mouse γ subunits were incubated with liposomes and the amount bound was determined as described in Materials and Methods. The composition of the liposomes (mol %) was 57% phosphatidylcholine (PC), 28% phosphatidylethanolamine (PE), and 15% PS (lanes 1, 3, 4, 6, 7, and 9) or 57% PC, 28% PE, 10% PS, and 5% PI-4-P (lanes 2, 5, and 8). Myristoylated Arf1, preloaded with GTPγS, was included in the liposomes analyzed in lanes 3, 6, and 9. Histograms show the amount (average ± standard deviation from three to four experiments) of AP-1 core cosedimented with liposomes (expressed as percent of total). In the absence of liposomes or the presence of liposomes containing PC and PE only, the amount of AP-1 in the pellet was <1–2%.

CHAPTER 4

PI(4)P Recruits GGA to the *trans* Golgi Network and Activates GGA Binding to Ubiquitin

Introduction:

The monomeric Golgi-localizing, γ-ear domain homology, Arf-binding proteins (GGAs) mediate TGN to endosome trafficking (Bonifacino, 2004; Ghosh and Kornfeld, 2003; Nakayama and Wakatsuki, 2003). Humans have three GGA genes, encoding GGA1, GGA2 and GGA3. They have three linearly arranged domains, called VHS (Vps27, Hrs, STAM), GAT (GGA and Tom1), and appendage (Figure 16A).

All human GGAs sort transmembrane cargoes that contain the acidic cluster-dileucine motifs by binding through their N-terminal VHS domain (Doray et al., 2002; Puertollano et al., 2003). In addition, GGA1 and 3 have been implicated in the sorting of ubiquitinated cargoes from the TGN (Bilodeau et al., 2004; Scott et al., 2004) and from endosomes (Puertollano and Bonifacino, 2004), by binding ubiquitin through their GAT domain (Bilodeau et al., 2004; Puertollano and Bonifacino, 2004; Shiba et al., 2004). GAT is composed of four helices, and the C-terminal "triple helix bundle" is a poly-functional module that interacts with multiple partners including ubiquitin (Mattera et al., 2004; Puertollano and Bonifacino, 2004; Shiba et al., 2004). Using affinity chromatography and nuclear magnetic resonance spectroscopy (NMR), Piper and colleagues found that the human GGA3 GAT domain contains "two" Ub binding motifs that bind to the same surface of ubiquitin (Bilodeau et al., 2004). These motifs are within different helices of the C-terminal "triple helix bundle". However, recently reported crystal structure of the GAT domain of human GGA3 in a 1:1 complex with ubiquitin reveals that only one hydrophobic and

acidic patch on helices α2 and α3 of the GAT "triple helix bundle" are ubiquitin binding sites (Prag et al., 2005) (Figure 16B).

Furthermore, GGA association with the TGN is dependent on the activation of the small GTPase Arf1 by GTP (Collins et al., 2003; Puertollano et al., 2001; Takatsu et al., 2002) and GGA's Arf1 binding site is located at the NH_2-terminus of GAT, upstream of the GAT ubiquitin binding sites (Shiba et al., 2003). EpsinR is a cargo-specific adaptor that cooperates with AP-1 to sort the SNARE protein Vti1b (Hirst et al., 2004) and regulates retrograde sorting on early endosome membranes (Saint-Pol et al., 2004). Like GGAs, it is also dependent on Arf1 for Golgi recruitment (Hirst et al., 2003; Mills et al., 2003).

In spite of the requirement for Arf1-GTP, GGAs and epsinR are unlikely to be recruited to the TGN exclusively by Arf1, because Arf1 has a broader intracellular distribution than these adaptors. As discussed in previous chapters, the major Golgi clathrin adaptor, AP-1, binds phosphatidylinositol 4 phosphate [PI(4)P] and requires PI(4)P for TGN targeting (Heldwein et al., 2004; Wang et al., 2003). In addition, other Arf1-dependent, TGN-localized proteins, such as epsinR (Hirst et al., 2003; Mills et al., 2003), OSBP (Levine and Munro, 2002) and FAPP (Godi et al., 2004; Stefan et al., 2002), that bind PI(4)P have also been identified.

Since PI(4)P is particularly enriched at the TGN (Wang et al., 2003), we and others have hypothesized that PI(4)P establishes TGN's organelle identity to facilitate the organelle-specific recruitment of trafficking adaptors (Munro, 2004; Simonsen et al., 2001; Wang et al., 2003). We found that AP-1 binds PI(4)P and its association with the TGN is PI(4)P dependent (Heldwein et al., 2004; Wang et al., 2003). However, the role of PI(4)P in the recruitment of the GGAs and epsinR has not been examined and the possibility that PI(4)P may function as more than a scaffold for these adaptors has not been considered at all.

In this study, we established that GGA's association with the TGN is dually regulated by PI(4)P and Arf1. PI(4)P also promotes GAT binding to monoubiquitin. Taken together, our results suggest that PI(4)P and Arf1 are essential components of a "coincidence detection network" that specifies recruitment of adaptors and cargoes through multiple low affinity interactions, and that PI(4)P is also a direct regulator GGA binding to ubiquitinated cargoes.

Results

GGA's TGN Targeting Is PI(4)P Dependent

As discussed previously, PI4KIIα is a Golgi resident kinase that accounts for a substantial fraction of the Golgi's PI(4)P (Wang et al., 2003). We showed previously that PI4KIIα knockdown by RNA interference (RNAi) decreases the amount of PI(4)P in the Golgi and blocks AP-1 recruitment to the TGN. We now find that PI4KIIα RNAi decreases TGN association of all three mammalian GGAs. This was demonstrated by using isoform-specific antibodies to localize endogenous GGA1 and GGA3, and by using overexpression to detect myc-tagged GGA2. Normally, GGAs are concentrated at the TGN and colocalize with TGN46, a TGN marker (Figure 15A). PI4KIIα RNAi decreases GGA association with the TGN, and increases cytosolic GGA. For example, GGA1 has a predominantly diffuse distribution in three of the four cells shown in Figure 15A. The cell that has GGA1 in the TGN may have less complete PI4KIIα knockdown than others in the field, and its GGA1 staining intensity at the TGN is less intense staining than cells not treated with PI4KIIα. As reported previously (Wang et al., 2003), the TGN is expanded in some RNAi cells, which is consistent with a block in export.

Likewise, epsinR is also mislocalized after PI4KIIα RNAi. This is demonstrated for both endogenous epsinR (Figure 15B, left panel) and overexpressed GFP-epsinR (Figure 15B, right panel). The epsinR response is similar to that of γ-adaptin, a subunit of the AP-1 adaptor complex.

It has been shown previously that GGAs, AP-1 and epsinR all bind GTP-activated Arf1 and their Golgi localization is dependent on Arf1-GTP (Bonifacino and Lippincott-Schwartz, 2003). Since PI4KIIα RNAi prevents their TGN association, it is important to rule out a direct effect of PI4KIIα RNAi on Arf1. Immunofluorescence studies show that Arf1 is enriched in the perinuclear region of PI4KIIα RNAi cells, although the area stained is somewhat broadened (Figure 14C) to a similar extent as TGN46. Since only activated Arf1 binds the Golgi and TGN, we conclude that PI4KIIα RNAi does not prevent Arf1 activation and that it disrupts TGN targeting of all adaptors by depleting PI(4)P from the TGN. Therefore, PI(4)P is required for the TGN association of almost all known TGN adaptors.

GGA Binds PI(4)P Through Its GAT Domain

GGA has four functionally distinct modules (Figure 16A), and previous studies have shown that its GAT domain binds Arf1, and GAT is sufficient for TGN targeting (Collins et al., 2003; Puertollano et al., 2001; Takatsu et al., 2002). Because of GGA's PI(4)P requirement for *in vivo* recruitment, we determined if GAT also binds PI(4)P. The lipid:protein overlay assay (Wang et al., 2003) shows that purified recombinant GGA2 VHS-GAT and GAT preferentially bind PI(4)P, compared with several other phosphoinositides and phosphatidylinositol (PI) (Figure 16C, top). Although VHS alone binds minimally, VHS linked to GAT (VHS-GAT)

binds PI(4)P slightly better than GAT alone. Therefore, VHS may promote GAT interaction with PI(4)P.

GAT binding to PI(4)P was also demonstrated by cosedimentation with liposomes (Heldwein et al., 2004). GGA1 and GGA2 GATs bind PI(4)P containing liposomes in a dose dependent manner (Figure 16C, bottom). There is much less binding to PS containing liposomes (Figure 17B) and almost no binding in the absence of acidic phospholipid (i.e. PC/PE only) (data not shown). These results establish that GGA1 and GGA2 GATs have a higher affinity for PI(4)P than PS. GGA1 and GGA2 bind PI(4)P to a similar extent (Figure 16C), suggesting that they bind PI(4)P with comparable affinities.

Since GGA association with the TGN is PI(4)P dependent, we ask if PI(4)P binding is necessary for GGA's association with the TGN. We approach this question by first identifying the minimal GAT domain that binds PI(4)P and using site-directed mutagenesis to generate PI(4)P-binding mutants for *in vitro* and *in vivo* analyses.

Identification of GAT's Minimal PI(4)P Binding Domain

GGA2 N-GAT and C-GAT domains (see Figure 16B for definition) were generated as GST-fusion proteins. N-GAT is a hook-like structure that encompasses the short helix α1 and the N-terminal half of long helix α2 (Collins et al., 2003; Shiba et al., 2003; Suer et al., 2003) (Figure 15B). It does not bind PI(4)P in protein:lipid overlay assays (data not shown).

We therefore focused on C-GAT, which is a three helix bundle consisting of the C-terminal half of helix α2, and the entire α3 and α4 (Figure 16B). Under our conditions, most of the bacterially expressed GST C-GAT, which should have an estimated molecular weight of 44 kDa, is spontaneously cleaved into multiple 25-32 kDa fragments (Figure 17A, top). Blotting

with anti-GST shows that some of these fragments contain the N-terminal GST tag (data not shown), and that their estimated sizes correspond to that of GST alone (28 kDa), GST-C terminal α2 (30 kDa) and GST-C terminal α2+3 (32 kDa). These results suggest that C-GAT may be preferentially cleaved at the flexible regions that link the helices. Individual helices without the GST tag (e.g. α4) are too small (approximately 2-3 kDa) to be retained in the SDS gel.

Unexpectedly, this mixture of C-GAT fragments binds PI(4)P significantly better than full-length GAT (Figure 17A, bottom). Increased binding may be explained by postulating that the fragments spontaneously assemble into a stable three helix bundle that binds PI(4)P, and that binding is improved relative to full-length GAT because N-GAT sterically blocks the COOH-terminal PI(4)P binding domain.

To further define the minimal C-GAT domain necessary for PI(4)P binding, GGA2 helices 3 and 4 were expressed individually and in tandem (α3+4). α3+4 bind PI(4)P to a similar extent as GAT, while α3 or α4 individually binds poorly (Figure 17A, bottom).

Identification of GGA's PI(4)P Binding Residues

Phosphoinositides bind proteins through multiple interactions, including contacts between their negatively charged phosphate headgroups and the side chains of basic amino acid residues (Collins et al., 2003; Heldwein et al., 2004; Yu et al., 2004). In some cases, the PI(4)P binding residues are organized into well-defined structural motifs, such as the pleckstrin homology (PH) domain (Yu et al., 2004) that is found in FAPP (Godi et al., 2004) and OSBP (Levine and Munro, 2002) or the ENTH domain found in epsinR (Hirst et al., 2003; Mao et al., 2001) and AP180 (Mao et al., 2001). In other cases, recognition occurs at regions with a cluster of basic residues that are part of a larger structure, as in AP-1 (Heldwein et al., 2004) and AP-2

(Collins et al., 2003). GGA GAT α3 and α4 do not have sequence or structural similarity to these phosphoinositide binding domains, but they have three basic residues that are almost completely conserved throughout phylogeny and are solvent exposed (Figure 16B). These and a few adjacent residues were mutated individually in the context of GAT. Protein:lipid overlay assays show that GGA2 GAT R276E, R281E, Y310E bind PI(4)P less well than wt GAT (Figure 17B, top panel), while K311E binds normally (data not shown).

Altered PI(4)P binding was confirmed by decreased cosedimentation with liposomes (Figure 17B, bottom). Wt GGA2 GAT binds PS, probably due to electrostatic interaction with the acidic lipid. Binding is increased 7.7X when PS was replaced with PI(4)P which is also acidic, establishing that GAT preferentially binds PI(4)P. Although the mutant GATs bind PS to a similar extent as wt GAT, they bind PI(4)P much less strongly. As a result, the PI(4)P/PS binding ratio decreases from 7.7X for wt GAT to 2.0, 2.0 and 1.2, respectively for GAT R276E, R281E and Y310E. Therefore, PI(4)P binding is inhibited without compromising GAT's overall affinity for acidic lipids.

Since GGA binding to the TGN is dependent on GTP-activated Arf1, we also examined the effect of these mutations on GAT interaction with Arf1. As expected, myristoylated Arf1-GTPγS promotes wt GGA2 GAT binding to PS liposomes. GGA2 GAT R276E and R281E are also activated by Arf1 to bind PS (1.7X increase) (Figure 17B, bottom), suggesting that they are able to interact productively with Arf1-GTP. This is expected, since GAT's Arf1 binding interface is in N-GAT (Shiba et al., 2003) (Figure 16B). In contrast, GAT Y310E responds poorly to Arf1 (1.2X increase), even though it is also not located at the Arf1 binding interface. GAT Y310E's coordinate loss of the Arf1 response and PI(4)P binding suggests that there is a global disruption of the GAT structure, which is consistent with the possibility that Y310

contributes to the packing of the three helix bundle (Shiba et al., 2004). On the other hand, the selective loss of PI(4)P but not Arf1 binding by GAT 276E or R281E suggests that these mutant GATs are unlikely to be disrupted structurally.

PI(4)P Binding Site Mutants Have Decreased Association With the Golgi

We first examined the *in vivo* consequences of mutation in the PI(4)P binding region in the context of GAT. HeLa cells were transfected with myc-tagged constructs encoding wt or mutant GGA2 GAT. As reported previously (Hirst et al., 2000; Puertollano et al., 2001), most of the tagged wt GGA2 GAT concentrates in a perinuclear region that colocalizes with TGN46 (Figure 18A). GAT2 K311E, which binds PI(4)P *in vitro*, is also enriched in the TGN. In contrast, GAT2 R276E and R281E are almost completely cytosolic, even at very low level expression (e.g. see cells in R281A panel). The lack of TGN association is not simply due to GAT charge reversal (from basic to acidic), because mutating R to A (basic to neutral) also prevents association (Figure 18A). The strong correlation between PI(4)P binding *in vitro* and TGN association *in vivo* supports a cause and effect relation between these two parameters.

Since GGAs have multiple interaction domains outside of GAT that can potentially contribute to TGN targeting, we also examined the behavior of these mutations in the context of full length GGA. At low level overexpression, wt GGA2 is predominantly TGN associated. In contrast, GGA2 R276A and R281A are found mostly in the cytoplasm (Figure 18B). However, in general, compared with mutant GATs, mutant GGAs are slightly more TGN-associated, suggesting that the ligand interactions mediated by non-GAT domains may contribute, albeit to a small extent, to TGN targeting. Nevertheless, since mutant GGAs are much less TGN associated than wt GGAs, PI(4)P binding is a major determinant of correct targeting of full-length GGAs.

PI(4)P Binding GGA Mutants Are Functionally Defective

We examined the functional consequences of the lack of PI(4)P binding and/or TGN localization by testing the ability of the PI(4)P binding mutants to rescue the abnormal TGN phenotype found in cells depleted of their endogenous GGA by RNAi (Ghosh et al., 2003; Ghosh and Kornfeld, 2003). We used GGA1 knockdown, because we have an antibody that detects endogenous GGA1. Western blotting confirmed that GGA1 was significantly decreased in cells exposed to GGA1 siRNA (Figure 18C').

The majority of control RNAi cells have compact perinuclear TGN46 staining, and only 12% have expanded TGN46 staining (Figure 18C", left panel). As reported previously (Ghosh et al., 2003), GGA1 RNAi induces abnormal TGN tubulation/expansion (in 50% of cells) (Figure 18C", right panel), which is attributed to an arrest of TGN export. Also, as reported previously (Ghosh et al., 2003), GGA1 overexpression decreases the percent of cells with enlarged Golgi to control level (11%).

In contrast, GGA1 R260A, at a comparable level of overexpression, is not concentrated in the Golgi and is unable to rescue the expanded Golgi phenotype (42% abnormal Golgi). Likewise, GGA1 R265A is also not effective (46% abnormal Golgi; data not shown in figure). Therefore, these PI(4)P binding mutants, like GGA2 R276A and R281A, have defective Golgi association and they are functionally defective.

PI(4)P Binding Is Necessary but not Sufficient for GGA Recruitment to the TGN. Dual Requirements for PI(4)P and Arf1.

Having established that PI(4)P binding is necessary for Golgi association by GGAs, we next determined if PI(4)P binding *per se* is sufficient. GGA2 C-GAT, which binds PI(4)P but not Arf1, has a diffuse distribution when overexpressed in cells (Figure 19). This confirms previous reports (Table 1) and establishes that PI(4)P binding is necessary but not sufficient for TGN targeting, and that N-GAT is required.

N-GAT binds Arf1, and Arf1 binding is previously reported to be necessary and sufficient for GAT association with the TGN (Collins et al., 2003; Puertollano et al., 2001). However, since our study highlighted the importance of PI(4)P to GGA association with the Golgi, and the N-GAT we used does not bind PI(4)P *in vitro*, this issue was reexamined further here. We found that GGA2 N-GAT is not concentrated in the perinuclear region (Figure 19), establishing that Arf1 binding alone is not sufficient for TGN association.

This conclusion contradicts that of Collins et al. (2003), who reported that GGA1 GAT165-234, which does not contain either of the PI(4)P binding residues we identified here, is recruited to Golgi membranes (Table 4). Their result can perhaps be explained by the fact that TGN recruitment was examined by adding recombinant proteins to permeabilized cells in the presence of cytosol, GTPγS and an ATP-regenerating system, instead of overexpression *in vivo*. It is possible that under the artificial *in vitro* conditions, GGA may have a less stringent requirement for PI(4)P because Arf1 is arrested in the GTPγS activated state. Our results clearly show that in living cells, where Arf1 cycles between the activated and inactivated states, Arf1 binding is not sufficient for TGN localization. In another study that uses overexpression (Puertollano et al., 2001), the GGA3 GAT147-260 fragment (equivalent to GGA2 164-278 or GGA1 147-261) tested contains a PI(4)P binding residue (Table 4). It can therefore theoretically bind Arf1 and PI(4)P, partially satisfying the dual requirements for Golgi association.

Relation Between PI(4)P and Ubiquitin Binding

The PI(4)P binding residues we identified are located in the GAT three helix bundle, a polyfunctional module that also binds ubiquitin (Figure 16B). Mammalian GGA1 and 3 and yeast GGAs all bind ubiquitin *in vitro*, while mammalian GGA2 binds much more weakly (Puertollano and Bonifacino, 2004; Shiba et al., 2004). Recently reported crystal structure of the GAT domain of human GGA3 in a 1:1 complex with ubiquitin reveals that a hydrophobic and acidic patch on helices α2 and α3 of the GAT three-helix bundle are ubiquitin binding sites (Prag et al., 2005), although there is general agreement that several residues in GAT α4 are also important for ubiquitin binding (Bilodeau et al., 2004; Mattera et al., 2004; Puertollano and Bonifacino, 2004; Shiba et al., 2004) (Figure 16B). GGA1 R260 (in α3) appears to be particularly important because GGA1 VHS-GAT R260E does not interact with ubiquitin in yeast two hybrid (Mattera et al., 2004).

Because ubiquitin and PI(4)P both bind GAT, we examined the effect of PI(4)P on GAT binding to ubiquitin. Recombinant GST-GAT was preincubated with PI(4)P micelles, and then exposed to agarose beads that are covalently linked to either monoubiquitin (Ub-agarose) or protein A (Ctrl). Very little GGA GAT binds control protein A-agarose, either in the presence (Figure 20A) or absence (data not shown) of PI(4)P. More GGA1 GAT binds Ub-agarose than protein A-agarose. PI(4)P further increases binding in a dose-dependent manner (Figure 20A, left panel), reaching 2X of the initial value [no PI(4)P] at 20 μM PI(4)P. Significantly, GGA2 GAT, which binds ubiquitin much weaker than GGA1 GAT in the absence of PI(4)P, also increases ubiquitin binding, to 2.9X in the presence of 15 μM PI(4)P (Figure 20A, right panel).

We ruled out a direct interaction of PI(4)P with ubiquitin, because ubiquitin does not bind PI(4)P dots even at 5 times the amount used to detect VHS-GAT binding (Figure 20C). That PI(4)P promotes ubiquitin binding by acting directly on GAT is clearly established with GAT PI(4)P binding mutants. GGA1 GAT R265A mutant binds Ub-agarose to a similar extent as wt GAT in the absence of PI(4)P (Figure 20B), but unlike wt GAT, its association with Ub-agarose is not increased by PI(4)P. Therefore, PI(4)P enhancement of GAT binding to ubiquitin is correlated with PI(4)P binding to GAT.

GAT R260A does not bind ubiquitin at all. This agrees with prediction from previous yeast two hybrid studies using VHS-GAT R260E (Mattera et al., 2004) and clearly establishes that R260 in helix 3 is critical to ubiquitin binding. Since GGA1 R265A mutation inhibits PI(4)P binding but not ubiquitin binding, whereas R260A decreases both, PI(4)P and ubiquitin appears to have overlapping but nonidentical requirements for binding.

We used GGA ubiquitination to confirm our ubiquitin binding results independently. It has been shown previously that GGA binding to ubiquitin is correlated with GGA ubiquitination (Shiba et al., 2004), although the regulatory mechanism underlying this coupling has not been determined. HeLa cells were cotransfected with myc-wt or mutant GGA1 and HA-ubiquitin, and myc-GGA1 was immunoprecipitated with anti-myc. Western blotting of cell lysates and immunoprecipitates shows that wt and mutant myc-GGA1 were expressed at equivalent levels, and similar amounts were immunoprecipitated (data not shown). Furthermore, wt and R265A GGA1 are ubiquitinated to a similar extent, while R260A is considerably less ubiquitinated (Figure 20D).

In conclusion, our data confirm that GGA1 R265A interacts normally with the ubiquitin machinery both in terms of ubiquitin binding and ubiquitination, while R260A is defective in

both respects. These results rule out, at least in the case of GGA1 R265A, the possibility that its lack of TGN association is due to defective ubiquitination and/or ubiquitin binding. Therefore, lack of PI(4)P binding is a more likely cause of defective Golgi recruitment.

Discussion

The findings presented here establish that GGA1 and GGA2 GAT both bind PI(4)P *in vitro* and the association of all three mammalian GGAs with the TGN is PI(4)P dependent. Furthermore, epsinR, which binds PI(4)P, also needs PI(4)P for TGN targeting. We have already shown previously that AP-1 association with the TGN is also PI(4)P dependent (Heldwein et al., 2004; Wang et al., 2003). Therefore, PI(4)P is a major lipid scaffold for the recruitment of almost all of the currently known TGN-enriched adaptors. Although these adaptors bind PI(4)P with relatively low affinity, their ability to sense both Arf1 activation and PI(4)P content allows them to act as coincidence detectors that specifically associate with the TGN in a spatially and temporally appropriate manner.

We find that GGA1 and GGA2 bind PI(4)P with similar affinity. Therefore, GGA interaction with PI(4)P binding, like Arf1 binding, is a quintessential GGA function. This is in contrast to differential GGA1 and 2 binding to ubiquitin and rabaptin-5 (Puertollano and Bonifacino, 2004; Scott et al., 2004; Shiba et al., 2004; Zhai et al., 2003) and differential phosphorylation (Ghosh et al., 2003; McKay and Kahn, 2004). We report that GGAs bind PI(4)P though their C-GAT domains, and VHS and N-GAT, which do not bind PI(4)P, may promote (Figure 16C) or hinder (Figure 17A) PI(4)P binding, respectively. Similar modes of regulation has been described for ubiquitin, which is not surprising since ubiquitin and PI(4)P both interact with C-GAT (Puertollano et al., 2001; Puertollano et al., 2003; Shiba et al., 2004)

We identified two conserved basic residues in C-GAT that are critical for PI(4)P binding *in vitro*, for association with the TGN and for normal GGA functions *in vivo*. These residues, R260 and R265 (equivalent to GGA2 GAT R276 and R281), are located in α3 of GAT's three helix bundle. Mutation of either individually diminishes PI(4)P binding *in vitro* and Golgi targeting *in vivo*. The requirement for both residues suggests that either they bind the same PI(4)P, or that they bind separate PI(4)Ps rather weakly, but avidity is increased when multiple PI(4)Ps bind.

The X-ray crystal structure of GAT (Collins et al., 2003; Shiba et al., 2003; Suer et al., 2003) shows that GGA1 R260 and R265 are located on the solvent exposed face of the GAT three helix bundle (Figure 16B) and are therefore accessible to the charged phosphate headgroups on PI(4)P. However, GGA1 R260 and R265 are separated by 13.7 angstrom (A), which is at the upper limit of the span that can be reached by a single PI(4)P molecule; PI(4)P is estimated to be approximately 8 A in its longest dimension, and it together with two salt bridges can potentially span 14 A. Moreover, these residues project in different directions from the GAT helix bundle. Therefore, if they were to bind the same PI(4)P molecule, at least one residue will have to move into sterically favorable interaction distance. Allosteric regulation of GGA by phosphorylation have been reported previously (Ghosh and Kornfeld, 2003; McKay and Kahn, 2004), and a PI(4)P-induced conformational change is not precluded by the current structure of the unliganded GAT.

Irrespective of how GGA binds PI(4)P, it is remarkable that despite GGA's engagement in the extensive network of parallel interactions at the Golgi, a single point mutation in GGA's PI(4)P headgroup interactive site is sufficient to almost completely prevent correct intracellular targeting. PI(4)P binding is therefore a critical upstream component of the GGA recruitment

cascade, and binding to Arf1 is not sufficient in its absence. A similarly stringent requirement has also been demonstrated recently for AP-1 (Heldwein et al., 2004; Wang et al., 2003). Therefore, PI(4)P is an important TGN scaffold that helps define the TGN's organelle identity.

Our results also show for the first time that PI(4)P is more than a scaffold, because it also regulates GGA interaction with ubiquitin, which is found on some cargo proteins as a sorting signal. PI(4)P promotes GAT binding to ubiquitin in a dose dependent manner. Therefore, PI(4)P and ubiquitin bind GGAs noncompetitively. Furthermore, PI(4)P and ubiquitin may bind GGAs cooperatively, since GGA1 R260 and R265 are on helix α3 while the majority of ubiquitin binding residues are located more upstream (Prag et al., 2005). Binding of one ligand may confer entropic advantage to the binding of another ligand.

Although the mechanism underlying the coupling of ubiquitin binding and ubiquitination is unclear, we used GGA ubiquitination to confirm the ubiquitin binding results. Consistent with its inability to bind ubiquitin, R260A has a defect on ubiquitination as well. In contrast, wt and R265A are ubiquitinated in a similar degree. Therefore, R260 is the residue critical to both PI(4)P and ubiquitin binding, whereas R265 binds PI(4)P but not ubiquitin. PI(4)P and ubiquitin have overlapping but nonidentical requirement for GGA binding.

It is more difficult to explain why GGA1 GAT R260A mutation has such a dramatic effect on ubiquitin binding, because GAT has additional ubiquitin binding motifs in α2 and α4. Also, it is puzzling that ubiquitin and PI(4)P have overlapping binding site on GGA (R260, according to human GGA1 nomenclature), and yet PI(4)P promotes, rather than inhibits, wt GAT interaction with ubiquitin. This scenario is different from that proposed for ubiquitin and rabaptin 5, which compete for GAT binding (Mattera et al., 2003; Scott et al., 2004; Shiba et al., 2004). The exact molecular basis for PI(4)P and ubiquitin binding, the possibility of their

cooperative binding and the consequences of R260A mutation will have to await high resolution X-ray structures of GAT in complex with these ligands.

Since GGA2, which normally binds ubiquitin more weakly than GGA1 and GGA3 (Puertollano and Bonifacino, 2004; Shiba et al., 2004), is also activated by PI(4)P to bind ubiquitin, it can potentially sort ubiquitinated cargoes as well. Indeed, the different inherent affinities of the various GGAs for ubiquitin and their common activation by PI(4)P may allow fine tuned regulation of ubiquitinated vs. nonubiquitinated cargo sorting at the TGN. In conclusion, our results suggest that trafficking of an ubiquitinated cargo may be directly regulated by the same phosphoinositide that recruits its cognate adaptor to the appropriate organelle membrane. This, together with a recent report that phosphoinositides tether a ubiquitin ligase that promotes the sorting of biosynthetic cargoes to internal organelle membranes (Dunn et al., 2004), underscore the emerging multifaceted roles of phosphoinositides in regulating membrane trafficking (Simonsen et al., 2001; Wenk and De Camilli, 2004).

Materials and Methods

Antibodies and Reagents

Affinity purified rabbit polyclonal anti-PI4KIIα was generated as described previously (Wei et al., 2002). Polyclonal anti-GGA1 and anti-epsinR were generated by the Robinson lab. Other antibodies were purchased from commercial sources. Polyclonal antibodies: sheep anti-TGN46 (Serotec), goat anti-GST (Amersham), secondary antibodies (Jackson ImmunoResearch, Amersham or Santa Cruz Biotechnology). Monoclonal antibodies: anti-Arf (ABR), anti-myc (Covance), anti-GGA3 (BD transduction laboratories), anti-His (BD Transduction), anti-γ

adaptin (Sigma), and anti-actin (Sigma). Most other reagents were from Sigma, except as noted in the text.

Protein Expression and Purification

GST-tagged GGA fusion proteins were expressed in the *Escherichia coli* strain BL21 (Invitrogen) using standard protocols and induction by IPTG at 20 C. Recombinant proteins were isolated by lysing bacteria in BugBuster reagent (Novagen) with benzonase nuclease. Soluble proteins were collected by centrifugation at 14,000 rpm (JA-14 rotor) at 4 C for 20 min. Proteins in inclusion bodies were obtained by suspending the pellets in BugBuster reagent, digestion with lysozyme, and resuspension in 8 M urea. GST fusion proteins were collected on Glutathione Sepharose 4 Fast Flow (Amersham Biosciences) and eluted with glutathione elution buffer containing 10 mM reduced glutathione and 50 mM Tris HCl (pH 8.0).

Myristoylated Arf1 (myrArf1) was generated by coexpression of Arf1 and yeast N-myristoyltransferase in *E. Coli*, and purified as described previously (Heldwein et al., 2004). Purified recombinant his-monoubiquitin (his-Ub) was a kind gift of Dr. George DeMartino (Univ. Texas Southwestern).

Generation of GAT Truncated Fragments and GGA Mutants

The GGA2 VHS-GAT p-GEX-4T-1 bacterial expression vector used is as described by Collins et al., 2002. Truncated GGA2 fragments (VHS, GAT, N-GAT, C-GAT, GAT α3, α4 and α34) were generated by PCR and subcloned into BamH1-Not1 sites of pGEX-4T-1 vector for protein purification or BamH1-Sma1 sites of pCMV5/myc1 for overexpression in HeLa cells. The myc-GGA1 and myc-GGA2 mammalian overexpression plasmids were gifts of Dr. Juan

Bonifacino (NIH). Site-directed mutagenesis was performed using the QuickChange kit (Stratagene). PCRs were performed using an overlap extension by *Pfu* Turbo DNA polymerase with full-length GGAs or GAT domain constructs as templates. Mutations were confirmed by nucleotide sequencing analysis.

Cell Culture and Plasmid Transfections

HeLa cells were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES and 1 mM sodium pyruvate at 37 C. They were transiently transfected with lipofecAMINE (Invitrogen), and cells were processed for immunofluorescence 18-24 h later.

RNA Interference

The PI4KIIα siRNA primers used were as described previously (Wang et al., 2003). GGA1 RNAi and rescue was performed based on the method described by Ghosh et al. (2004), except that we used siRNA for knockdown (^{463}AAGCTTCCAGATGACACTACC^{483}, (AF190862)) and transient cDNA transfection for rescue. HeLa cells were plated in 6-well plates at between 30 to 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 at 72 h after the initial siRNA treatment. In the rescue experiments, RNAi cells were transfected with GGA1 cDNA 54 h after the initial siRNA transfection and harvested 18 h after cDNA transfection.

Cells were processed for immunofluorescence microscopy after fixing in methanol at -20 C for 10 min. Fixed and permeabilized cells were incubated with antibodies in PBS containing

1% BSA and 3% donkey serum. Immunofluorescence was detected by a Zeiss 510 Laser Scanning Confocal Microscope using a 63X 1.3 NA PlanApo objective.

Samples were analyzed by SDS polyacrylamide gel electrophoresis in 4-20% gradient gels, transfer onto nitrocellulose membranes and immunoblotted with the indicated antibodies. Blots were developed with the ECL detection kit (Amersham Biosciences) and immunoreactive products were identified by exposure to Hyperfilm ECL.

Protein-Lipid Overlay Assay

The assay was as described previously (Wang et al., 2003). PIP-Arrays (Echelon Biosciences) or "homemade" lipid strips were blocked in TBST buffer containing 10 mM Tris (PH 8.0), 150 mM NaCl, and 0.1% (v/v) Tween 20 and 3% fatty acid free BSA for 1 h at room temperature, followed by incubating with purified recombinant GST-tagged GGA fragments or His-tagged ubiquitin at 4 C overnight. Membranes were washed extensively with TBST (without BSA) and probed with primary and secondary antibodies conjugated to HRP. Bound immunocomplexes were identified with ECL Plus followed by phosphorimager analyses.

***In Vivo* Ubiquitination**

COS cells grown on 10 cm dishes were transfected myc-tagged GGA1 wt or mutant cDNA and HA-tagged monoubiquitin cDNA (a gift from Dr. Kim Orth, Univ. Texas Southwestern). 24 h after transfection, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP-40, 0.1% SDS, 0.5% DOC and protease inhibitors) and centrifuged at 14,000 x g for 20 min at 4 C. The supernatant was incubated with anti-myc antibody at 4 C overnight with tumbling, followed by incubation with protein G

agarose for 1 hr. Beads were washed successively with high salt buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.025% NaN_3) and low salt buffer (50 mM Tris HCl, pH 6.8). Immunoprecipitated proteins were subjected to SDS polyacrylamide gel electrophoresis and detected by western blotting.

Liposome Binding Assay

Mixed lipid vesicles were prepared as described in Heldwein et al. (Heldwein et al., 2004). Dioleoyl phosphatidylcholine (PC), dipalmitoyl phosphatidyl-ethanolamine (PE), brain phosphatidylserine (PS) and brain PI(4)P (all from Sigma) were dissolved in chloroform/methanol and the solvent was evaporated by exposing to a stream of nitrogen, followed by exposure to vacuum overnight. The lipid film was resuspended by vortexing at room temperature in Tris buffer (Tris HCl, pH 7.5, 50 mM NaCl and 1 mM DTT) to a final concentration of 1 mg/ml. 0.02 mg/ml (0.4 μM) recombinant GATs were incubated with lipid vesicles (usually 0.2 mg/ml) for 15 min. at room temperature in a final volume of 75 μl in Tris buffer.

In some cases, GAT was incubated with liposomes precharged with myrArf1-GTPγS. These vesicles were prepared by incubating 8 μM myrArf1 in Tris buffer containing 1 mM $MgCl_2$ and 2 mM EDTA and 40 μM GTPγS for 45 min at 37 C prior to liposome addition. Vesicles were collected by centrifugation, resuspended in Tris buffer supplemented with 1 mM $MgCl_2$, and then exposed to GAT. All samples were centrifuged for 10 min. at 200,000 x g (TL100, Beckman Coulter) at 25 C. The pellets were resuspended with Tris buffer and subjected to SDS polyacrylamide gel electrophoresis. Bound proteins were detected by Coomassie blue staining and densitometry.

Ubiquitin Binding Assay

PI(4)P micelles were prepared by sonicating PI(4)P (Echelon Biosciences) as described previously (Lin et al., 1997). 0.5 □M GST-tagged GAT was incubated with PI(4)P micelles for 15 min at room temperature in buffer A (25 mM Hepes, pH 7.4, 125 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, and 1 mM DTT) in a 100 μl volume. 15 μl ubiquitin- or protein A-agarose beads (Sigma) that were preincubated with buffer A containing 0.1% BSA were added and incubation was continued for 60 min. The beads were pelleted and washedγ five times with buffer A/1% Triton X-100. Bound GST-GAT was detected by western blotting with anti-GST.

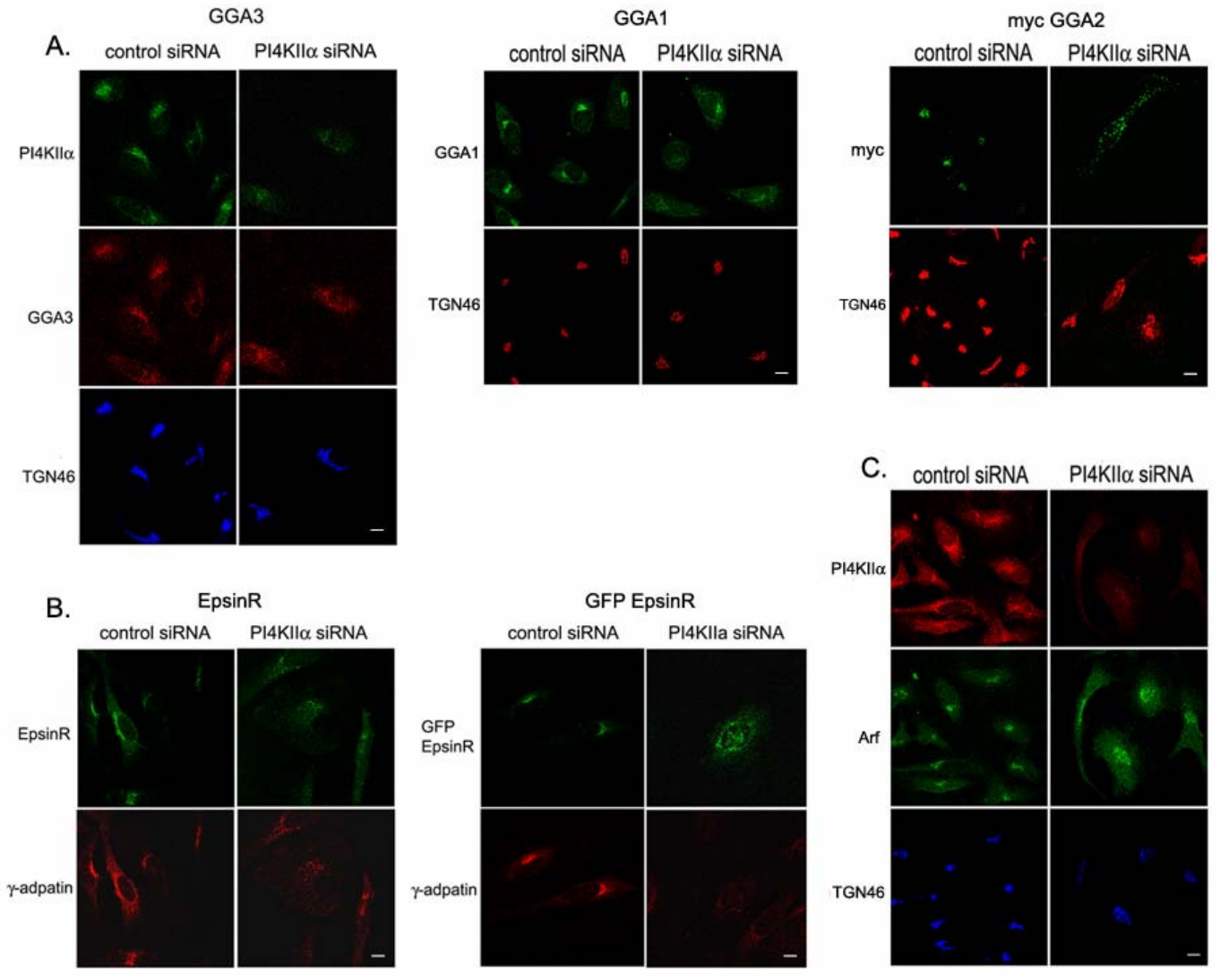

A.
GGA3
control siRNA
PI4KIIα siRNA
PI4KIIα
GGA3
TGN46
GGA1
control siRNA
PI4KIIα siRNA
GGA1
TGN46
myc GGA2
control siRNA
PI4KIIα siRNA
myc
TGN46
B.
EpsinR
control siRNA
PI4KIIα siRNA
EpsinR
γ-adpatin
GFP EpsinR
control siRNA
PI4KIIa siRNA
GFP EpsinR
γ-adpatin
C.
control siRNA
PI4KIIα siRNA
PI4KIIα
Arf
TGN46

Figure 15. Effects of PI4KIIα RNAi on GGA, epsinR and Arf1 distribution. Hela cells were transfected with control or PI4KIIα siRNA and processed 72 h later for immunofluorescence microscopy. (A) GGAs. Cells were fixed and triple-labeled to detect PI4KIIα, TGN46 and endogenous GGA1 or GGA3. Effects on GGA2 distribution was determined by overexpressing myc-GGA2. PI4KIIα siRNA cells were transfected with myc-GGA2 cDNA 18 h prior to fixation. Cells were labeled with anti-myc. (B) EpsinR. Endogenous epsinR was detected with anti-epsinR, and GFP-epsinR was overexpressed after transient transfection. Endogenous γ-adaptin (a subunit of AP-1) was detected by antibody. (C) Arf1 localization. RNAi cells were triple stained with anti-PI4KIIα, anti-TGN46 and anti-Arf. The anti-Arf antibody recognizes all Arfs, but Arf1 is most abundant and only Arf1 is concentrated in the Golgi. Scale bar, 20 μm.

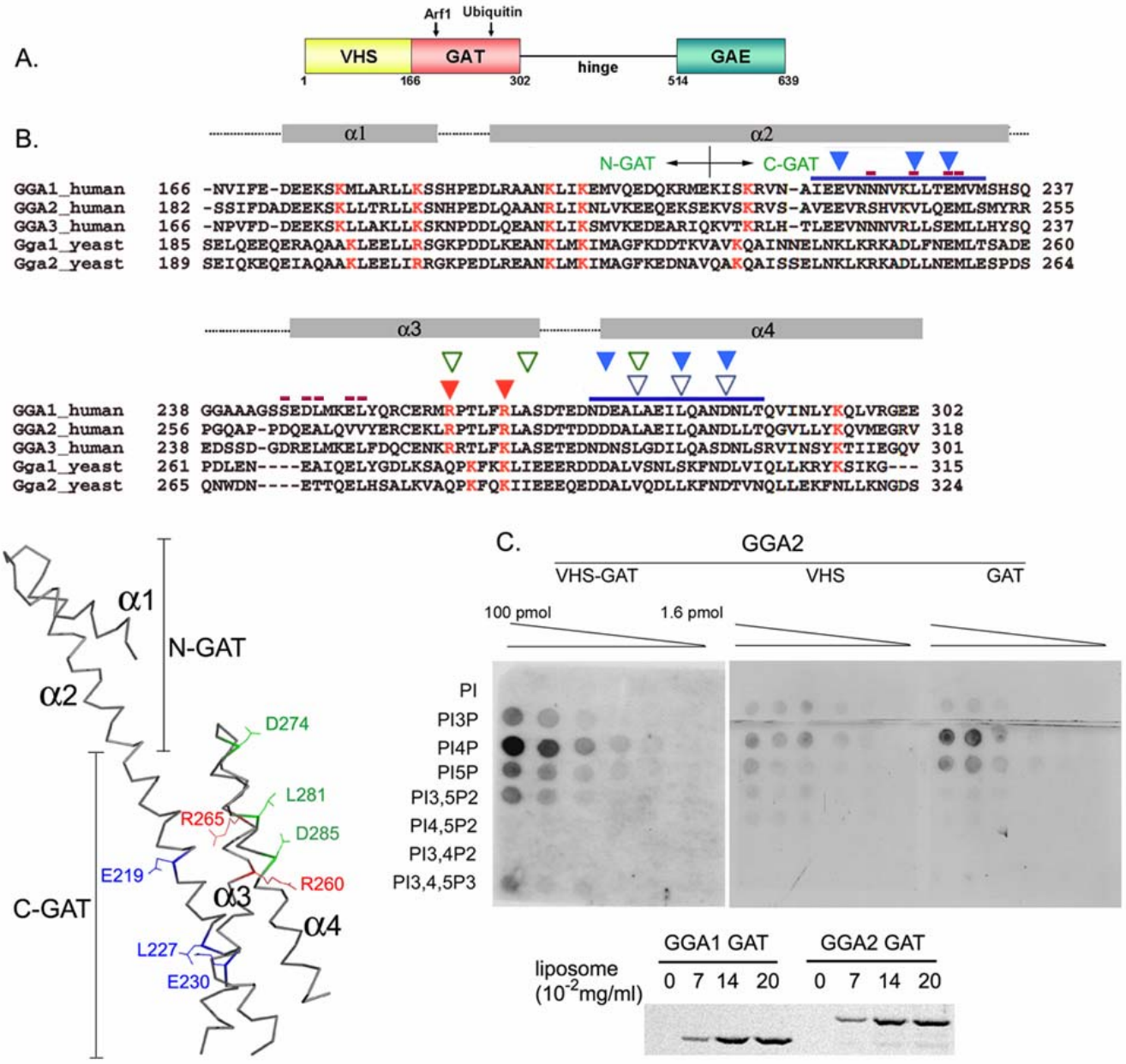

A.
Arf1
Ubiquitin
VHS
GAT
hinge
GAE
1
166
302
514
639
B.
α1
α2
N-GAT
C-GAT
GGA1_human 166 -NVIFE-DEEKSKMLARLLKSSHPEDLRAANKLIKEMVQEDQKRMEKISKRVN-AIEEVNNNVKLLTEMVMSHSQ 237
GGA2_human 182 -SSIFDADEEKSKLLTRLLKSNHPEDLQAANRLIKNLVKEEQEKSEKVSKRVS-AVEEVRSHVKVLQEMLSMYRR 255
GGA3_human 166 -NPVFD-DEEKSKLLAKLLKSKNPDDLQEANKLIKSMVKEDEARIQKVTKRLH-TLEEVNNNVRLLSEMLLHYSQ 237
Gga1_yeast 185 SELQEEQERAQAAKLEELLRSGKPDDLKEANKLMKIMAGFKDDTKVAVKQAINNELNKLKRKADLFNEMLTSADE 260
Gga2_yeast 189 SEIQKEQEIAQAAKLEELIRRGKPEDLREANKLMKIMAGFKEDNAVQAKQAISSELNKLKRKADLLNEMLESPDS 264
α3
α4
GGA1_human 238 GGAAAGSSEDLMKELYQRCERMRPTLFRLASDTEDNDEALAEILQANDNLTQVINLYKQLVRGEE 302
GGA2_human 256 PGQAP-PDQEALQVVYERCEKLRPTLFRLASDTTDDDDALAEILQANDLLTQGVLLYKQVMEGRV 318
GGA3_human 238 EDSSD-GDRELMKELFDQCENKRRTLFKLASETEDNDNSLGDILQASDNLSRVINSYKTIIEGQV 301
Gga1_yeast 261 PDLEN----EAIQELYGDLKSAQPKFKKLIEEERDDDALVSNLSKFNDLVIQLLKRYKSIKG--- 315
Gga2_yeast 265 QNWDN----ETTQELHSALKVAQPKFQKIIEEEQEDDALVQDLLKFNDTVNQLLEKFNLLKNGDS 324
α1
N-GAT
α2
D274
L281
R265
D285
E219
R260
C-GAT
α3
α4
L227
E230
C.
GGA2
VHS-GAT
VHS
GAT
100 pmol
1.6 pmol
PI
PI3P
PI4P
PI5P
PI3,5P2
PI4,5P2
PI3,4P2
PI3,4,5P3
GGA1 GAT
GGA2 GAT
liposome
(10-2mg/ml)
0 7 14 20
0 7 14 20

Figure 16. GGA GAT PI(4)P binding domain. (A) GGA domain organization. GGA has three functionally distinct modules named VHS, GAT, and GAE (γ-adaptin ear homology domain). Numbers indicate domain boundaries based on GGA1 sequence and as defined by Suer et al. (2003). GAT has four α-helices, and it binds Arf1 at the N-terminal hook region, and ubiquitin at the C-terminal triple helix bundle. (B) The GAT domain. Top, sequence alignment of human and yeast GGA GATs. Human has three GGA genes, and yeast has two. Rectangles, helices in GAT, dotted lines, linkers between helices. The boundary used to generate the N-terminal GAT (N-GAT) and C-GAT (triple helix bundle, α234) is indicated. Red letters denote conserved basic amino acids that can potentially bind PI(4)P. In some cases, they are offset by a maximum of two residues to optimize alignment. Red arrowheads indicate residues which we identified to be important for PI(4)P binding; green, open and filled blue arrowheads, residues implicated in ubiquitin binding by Shibat et al. (2003), Puertollano and Bonifacino (2004) and Bilodeau et al. (2004), respectively. Red lines, ubiquitin binding residues determined by X-ray crystallography (Prag et al, 2005). Blue lines, two independent ubiquitin binding motifs identified by Bilodeau et al. (2004) with NMR in yeast. Bottom, X-ray crystal structure of GGA1 GAT. The template was downloaded from the protein database; putative PI(4)P binding residues are indicated in red, and ubiquitin binding residues in motif 1 and 2 (as defined by Bilodeau et al. (2004) in blue and green, respectively. The flexible linkers between the helices were not shown. (C) Human GGA2 domain binding to lipids. Top, lipid overlay assay. PIP-Arrays (Echelon Biosciences) were incubated with 0.2 μg/ml purified recombinant GST-tagged human GGA2 VHS-GAT, VHS or GAT and bound proteins were detected with anti-GST. Bottom, GGA1 and GGA2 GAT binding to mixed liposomes. Increasing amounts of PC/PE/PI(4)P (60:30:10) liposomes were incubated with 0.5 μM GST-tagged GGA1 or GGA2 GAT. GST alone does not bind liposomes (data not shown).

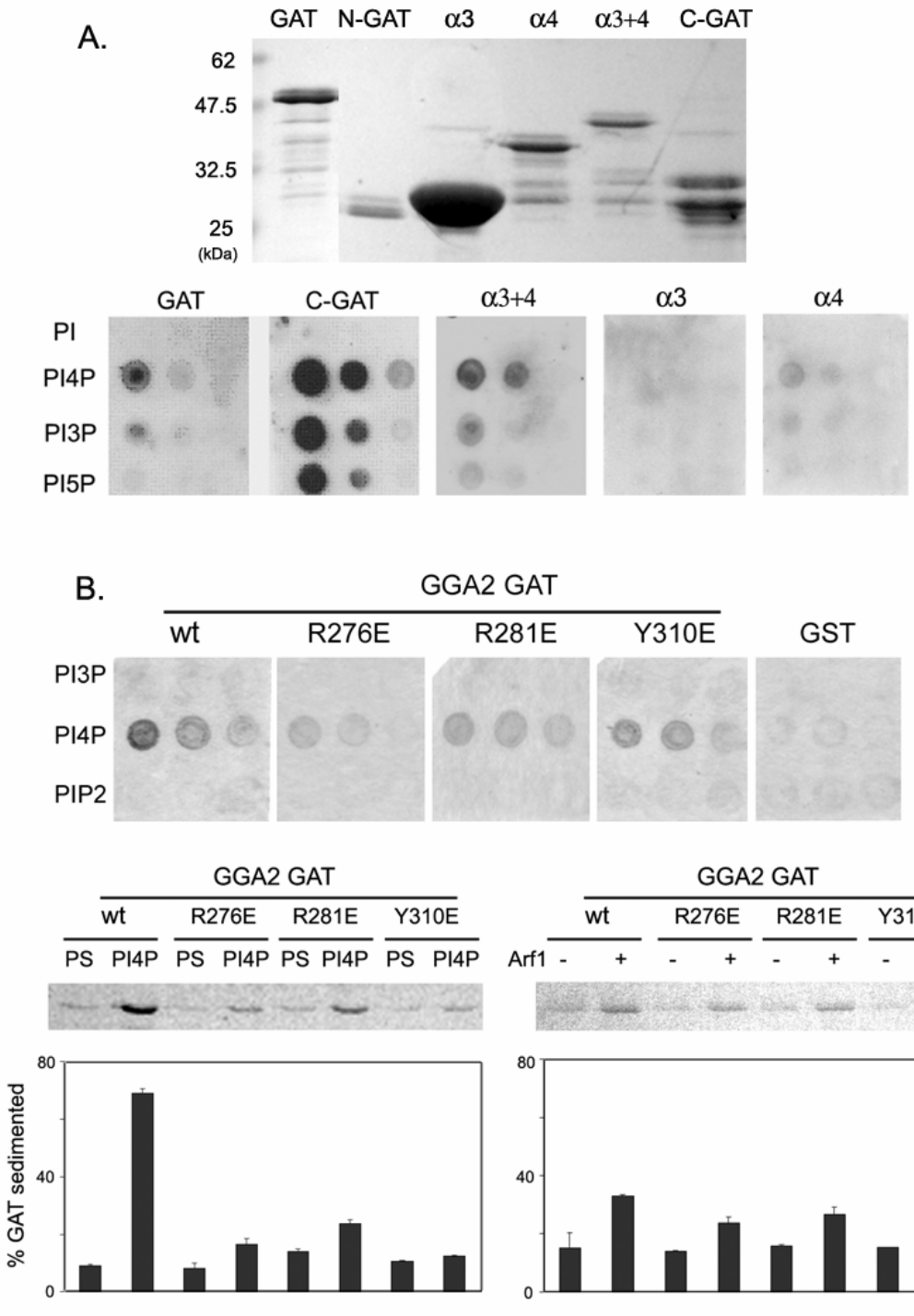

A.
GAT
N-GAT
α3
α4
α3+4
C-GAT
62
47.5
32.5
25
(kDa)
GAT
C-GAT
α3+4
α3
α4
PI
PI4P
PI3P
PI5P
B.
GGA2 GAT
wt
R276E
R281E
Y310E
GST
PI3P
PI4P
PIP2
GGA2 GAT
wt
R276E
R281E
Y310E
PS
PI4P
Arf1
% GAT sedimented
80
40
0

Figure 17. Identification of GAT PI(4)P binding residues. (A) The minimal PI(4)P binding domain. GGA2 GAT subdomains were expressed in *E. coli*, purified and analyzed by SDS-PAGE and coomassie blue staining (left). GAT subdomains (0.2 μg/ml, equivalent to 4 nM GAT) were incubated with membranes dotted with 100, 50 and 25 pmol lipids (right). (B) GGA2 PI(4)P binding mutants. GGA2 GAT residues were mutated to glutamic acid (E) by site-directed mutagenesis. Top, protein:lipid overlay assay; bottom, liposome binding. 0.4 μM human wt and mutant GGA2 GAT were incubated with 0.2 mg/ml mixed lipid vesicles. Left panel, PC/PE (57%:28%) liposomes containing 15% PS or PI(4)P. Right panel, 57% PC/28% PE/15% PS liposomes were preloaded with recombinant myrArf1-GTPγS (right panel). Liposomes were sedimented and bound proteins were detected by coomassie blue staining after SDS-PAGE. Histograms show the percent of total GAT sedimented, as mean±SE from two to four experiments.

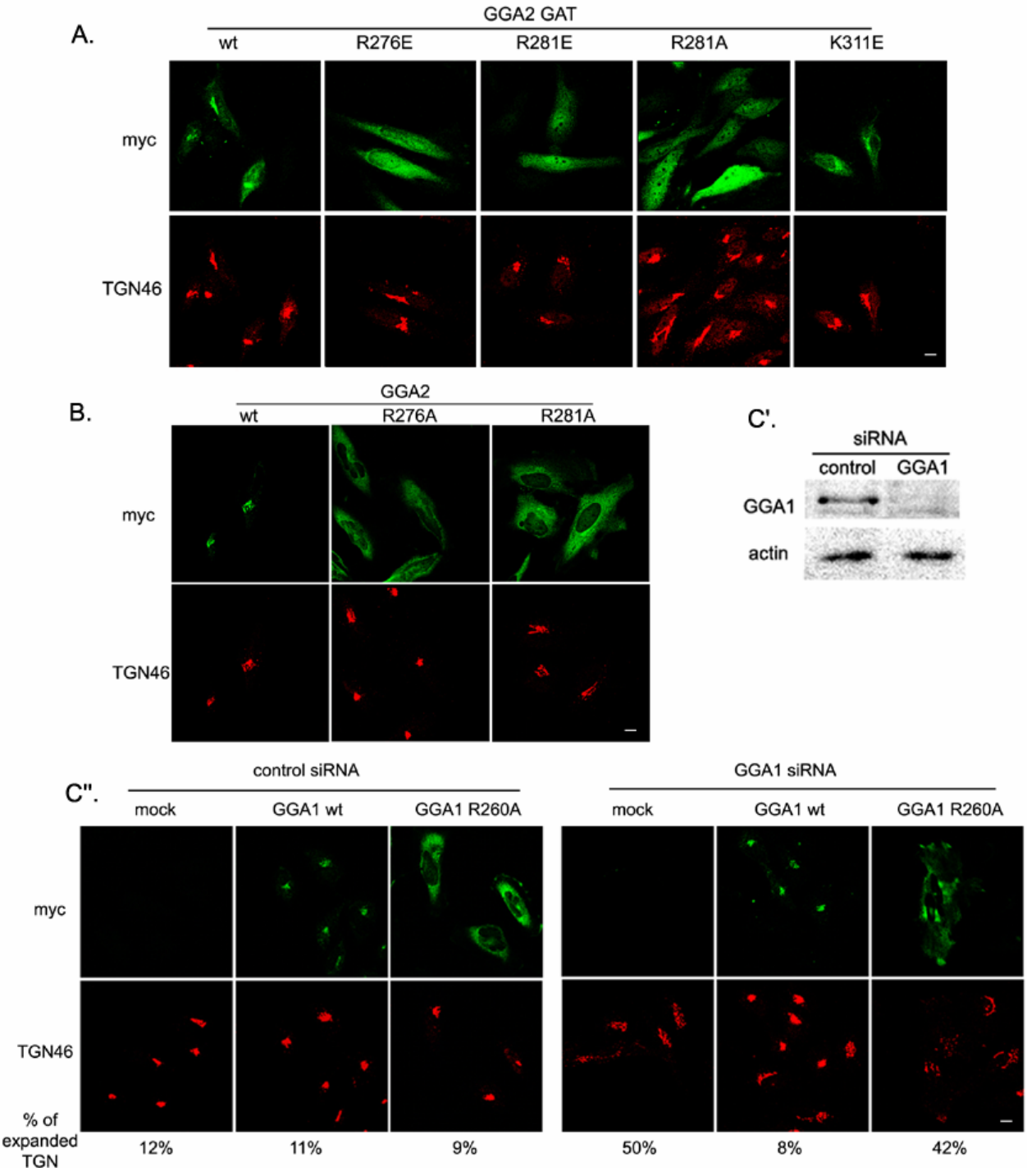

A.
GGA2 GAT
wt
R276E
R281E
R281A
K311E
myc
TGN46
B.
GGA2
wt
R276A
R281A
myc
TGN46
C'.
siRNA
control
GGA1
GGA1
actin
C''.
control siRNA
GGA1 siRNA
mock
GGA1 wt
GGA1 R260A
mock
GGA1 wt
GGA1 R260A
myc
TGN46
% of expanded TGN
12%
11%
9%
50%
8%
42%

Figure 18. PI(4)P binding mutants are not Golgi targeted and cannot rescue the GGA knockdown TGN phenotype. (A) GAT targeting. GGA2 myc-GAT wt, R276E, R281E, R281A, K311E were expressed in HeLa cells, and detected by staining with anti-myc. (B) Full-length GGA targeting. Myc-GGA2 wt and mutants were expressed in HeLa cells. (C) GGA1 RNAi rescue. C', western blot. HeLa cells transfected with control or GGA1 siRNA were harvested and lysates were subjected to western blotting with anti-GGA1 and anti-actin (as a loading control). C", effect of wt or mutant GGA1 overexpression on the GGA1 knockdown TGN phenotype. Control or GGA1 RNAi cells were transiently transfected with wild-type or mutant myc-GGA1 cDNA 60 h after siRNA transfection and cells were processed for immunofluorescence microscopy 18 h later. Cells in randomly chosen fields were scored for the presence of normal perinuclear TGN46 staining or expanded TGN46 phenotype. The percent of cells with expanded TGN46 is indicated at the bottom. Data is from a typical experiment, in which 150-200 transfected cells were scored per condition. Similar results were obtained in three independent experiments. Scale bar, 20 μm.

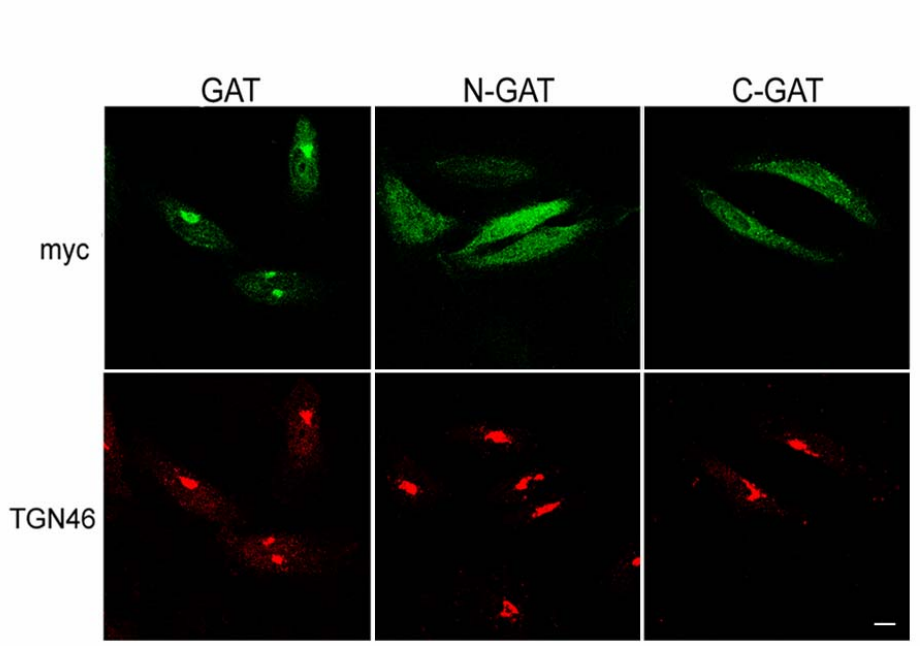

GAT
N-GAT
C-GAT
myc
TGN46

Figure 19. Neither PI(4)P nor Arf1 binding is sufficient for GGA association with the TGN. Full-length myc-GGA2 GAT, N-GAT and C-GAT were expressed in HeLa cells, and their intracellular distribution was determined by immunofluorescence staining with anti-myc.

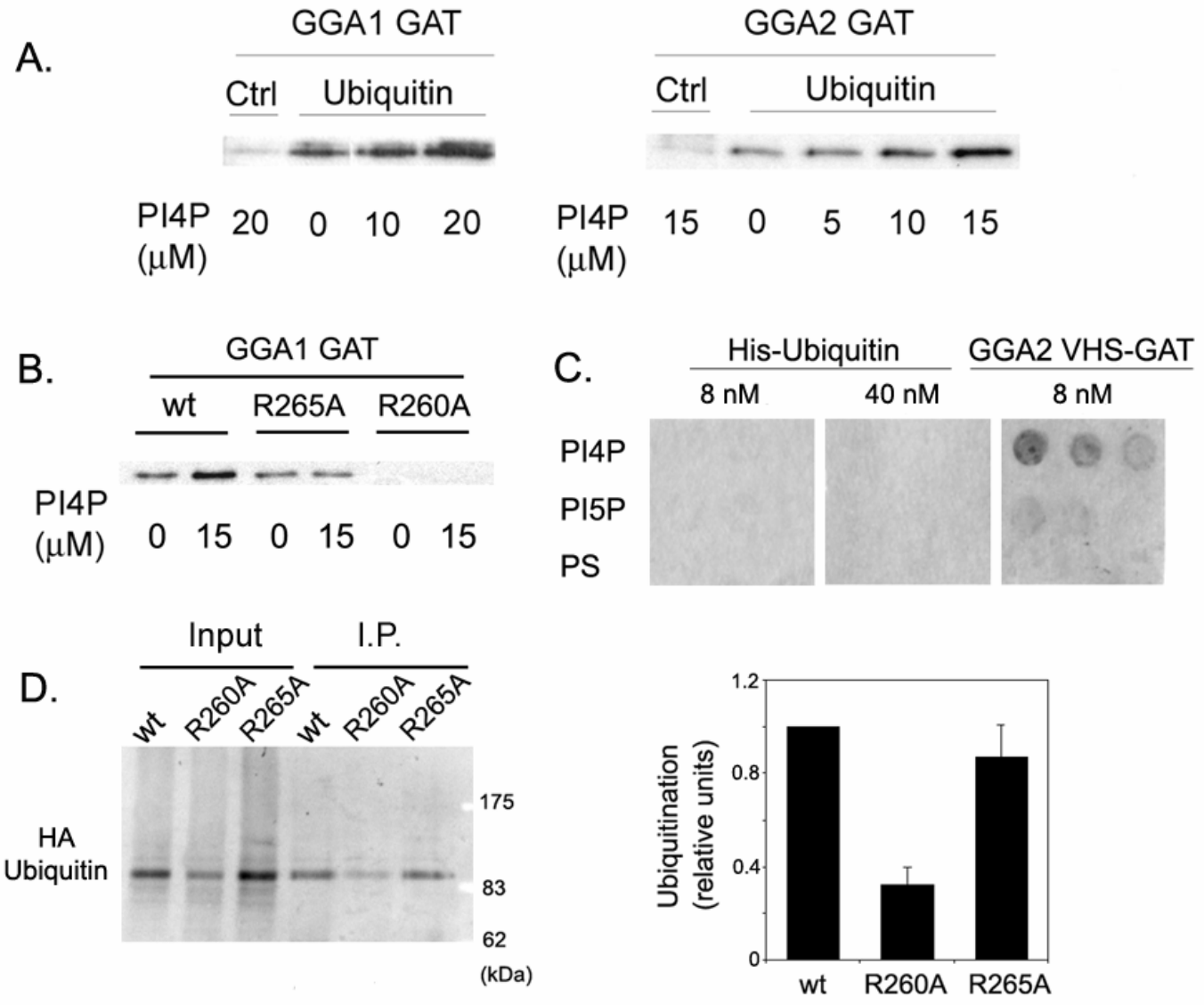

A.
GGA1 GAT
Ctrl
Ubiquitin
PI4P (μM) 20 0 10 20
GGA2 GAT
Ctrl
Ubiquitin
PI4P (μM) 15 0 5 10 15
B.
GGA1 GAT
wt
R265A
R260A
PI4P (μM) 0 15 0 15 0 15
C.
His-Ubiquitin
GGA2 VHS-GAT
8 nM
40 nM
8 nM
PI4P
PI5P
PS
D.
Input
I.P.
wt
R260A
R265A
wt
R260A
R265A
HA Ubiquitin
175
83
62
(kDa)
Ubiquitination (relative units)
1.2
0.8
0.4
0
wt
R260A
R265A

Figure 20. PI(4)P promotes GGA binding to ubiquitin. Recombinant GGAs were incubated with protein A-agarose (Ctrl) or Ub-agarose (ubiquitin) that were precoated with BSA in the absence of detergent. The beads were then extensively washed in the presence of detergent and bound GGA GAT was detected by western blotting with anti-tag antibody. (A) PI(4)P promotes ubiquitin binding by wt human GAT. 0.5 μM GGA1 or GGA2 GAT was used, and bound GAT was detected with anti-GST. (B) PI(4)P does not promote mutant GAT binding to ubiquitin. GGA1 GAT wt, R260A and R265A (0.25 μM) were preexposed to increasing amount of PI(4)P micelles and then incubated with Ub-agarose in the presence of PI(4)P. (C) Ubiquitin does not bind PI(4)P. His-monoubiquitin (at 8 or 40 nM) and GST-VHS-GAT (at 8 nM) were incubated with lipid dots (100, 50 and 25 pmol), and bound protein was detected with anti-his or anti-GST. (D) GGA ubiquitination. HeLa cells were cotransfected with myc-tagged wt or mutant GGA1 and HA-Ub cDNAs. Left panel, western blot. Cells were lysed and myc-GGA1 was immunoprecipitated with monoclonal anti-myc. Cell lysates (input) and immunoprecipitates (I.P.) were analyzed by SDS-PAGE and western blotted with monoclonal anti-myc and anti-HA to detect myc-GGA1 and HA-Ub, respectively. Right panel, quantitation of GGA1 ubiquitination. The extent of ubiquitination is expressed as a ratio of the intensity of the HA to myc signals in western blots of the GGA1 immunoprecipitates. The value for wt GGA1 is defined as 1. Data shown is mean±SE (n=3).

Table 4. Comparison of GGA GAT's requirement for Arf1, PI(4)P and TGN association

Construct	Equivalent to GGA1 residues	Arf1-GTP binding	TGN targeting	No. of PI(4)P binding residues
This study				
GGA2 GAT 181-331	165-314	+	+	2
GGA2 GAT 181-221, N-GAT	165-204	[+]	-	0
GGA2 GAT 222-331, C-GAT	205-314	[-]	-	2
GGA2 GAT 255- 331, α34	238-314	[-]	ND ?	2
GGA2 GAT 255-287, α3	238-271	[-]	ND	2
GGA2 GAT 288-331, α4	272-314	[-]	ND	0
Puertollano et al. (2001)				
GGA3 GAT 147-260	147-261	ND	+ (o/e)	1
Collins et al. (2003)				
GGA1 GAT 165-234	-	+	+ (*in vitro*)	0
GGA1 GAT 205-314	-	-	- (*in vitro*)	2

[ ], predicted; ND, not determined; (*in vitro*) refers to TGN recruitment assay with recombinant protein.

CHAPTER 5

Conclusions and Future Directions

The Golgi is the central sorting station for membrane trafficking, and phosphoinositides may be cofactors or modulators of multiple Golgi functions. Although PIP2 is firmly established as a cofactor in plasma membrane trafficking, its role in the Golgi is less definitive. In fact, yeast uses PI(4)P, and not PIP2, to regulate constitutive secretion from the late Golgi. This unexpected finding and the large amount of PI(4)P in the Golgi raise the distinct possibility that PI(4)P has an important *direct* role in the mammalian Golgi.

The goals of this dissertation are to gain more understanding about the functions of PI(4)P in the mammalian Golgi. We found that Golgi PI(4)P is generated primarily by a Golgi-resident phosphatidylinositol 4 kinase called PI4KIIα, which converts PI to PI(4)P. PI4KIIα RNAi knocks down PI4KIIα specifically and decreases the Golgi PI(4)P content by at least 60%. As a consequence, recruitment of clathrin adaptor AP-1 complex to the TGN was blocked. This AP-1 binding defect is a direct consequence of PI(4)P decrease, since the defect is rescued by adding back PI(4)P, but not PIP2. In addition, purified AP-1 binds PI(4)P but not PIP2 in protein-lipid overlay assay, and anti-PI(4)P inhibits the *in vitro* recruitment of cytosolic AP-1 to normal cellular membranes. Therefore, we propose that PI4KIIα establishes the TGN's unique lipid-defined organelle identity by generating PI(4)P-rich domains that specify the docking of the AP-1 coat machinery.

It is already widely accepted that clathrin adaptors TGN recruitment is regulated by GTP-bound small GTPase Arf1. However, paradoxically, Arf1 has a broader intracellular distribution than these adaptors. It is therefore unlikely to be the only docking site for adaptors.

Our finding that PI(4)P is another docking site for adaptor TGN recruitment provides important clues as to how adaptor recruitment is regulated. Our results imply that PI(4)P binding may specify TGN-specific recruitment in conjugation with Arf1. Our hypothesis is confirmed by others who showed that association of FAPPs with the TGN is dependent on PI(4)P and regulated by Arf1 (Godi et al., 2004). Therefore, "coincidence detection network" emerges as an appealing model: the interaction of cytosolic adaptor proteins with both lipid [PI(4)P] and protein (Arf1) on the TGN membrane is required to control their recruitment to TGN.

Although the attempt to cocrystalize AP-1 with phosphatidylinositide failed, we can deduce aspects of a potential interaction with PI(4)P by reference to the IP6 site in the AP-2 crystals. We found that three residules, Tyr-45, Arg-48 and Lys-52, at a corner of the gamma chain may be the potential PI(4)P binding sites. Site-directed mutations of these residues prevent GFP-γ AP recruitment to the TGN in cells. Furthermore, R48A diminishes PI(4)P-dependent, but not Arf1-dependent, liposome binding *in vitro*. We propose that these basic residues are important for AP-1 binding to PI(4)P, and this binding is essential for TGN targeting. In the absence of these contacts, Arf1 does not suffice.

We have also focused on the GGA clathrin adaptors. There are three mammalian GGAs, and they have three linearly arranged domains, called VHS, GAT, and appendage. We now show that knockdown of PI4KIIα decreases GGA association with the TGN. We found that VHS-GAT and GAT domains recombinant proteins bind PI(4)P directly but not PIP2 with lipid-protein overlay and liposome binding assays. By doing a series of deletion experiments, we located the PI(4)P binding domain at the C-terminal "triple helix bundle" of the GAT domain. Since GAT binds Arf1 at its N-terminal "hook helix", GAT can bind Arf1 and PI(4)P

simultaneously. However, neither the PI(4)P-binding domain nor the Arf1-binding domain alone is correctly targeted to the TGN.

Based on the published crystal structures of GGA1 GAT, we mutated conserved basic residues in a solvent exposed patch in the helix bundle, and identified two arginine residues that are critical for PI(4)P binding *in vitro* and TGN targeting *in vivo*. These mutations do not alter Arf1 binding. According to the crystal structure of GAT domain, these two arginine residues protrude from the helix and are likely to bind different PI(4)P molecules.

Further studies show that the PI(4)P-binding mutants of GGA1 are not functional *in vivo*. GGA1 RNA interference blocks TGN export and induces abnormal Golgi expansion phenotype. Overexpression of wild type GGA1 rescues the phenotype, but mutant GGA1s do not.

Having established that GGA binds PI(4)P, we sought to determine if PI(4)P regulates the GGAs function with other ligands. The C-terminal "triple helix bundle" of GAT domain is a polyfunctional module that also interacts with multiple partners including ubiquitin, which may provide a recognition signal for GGAs to control protein sorting. We found that PI(4)P increases wt GAT binding to ubiquitin-conjugated agarose beads, but has no effect on PI(4)P-binding mutant GAT. Therefore, PI(4)P may be an allosteric regulator for GGAs to enhance ubiquitin binding to GGAs.

Based on these results, we conclude: (1) PI(4)P defines the TGN organelle identity by recruiting TGN-targeted adaptors; (2) TGN-enriched adaptors are recruited to the Golgi by binding to both PI(4)P and Arf1, and neither is sufficient; (3) Besides acting as a scaffold, PI(4)P may also be an allosteric regulator for GGAs that modulates GGA function with other ligands, such as ubiquitin.

The finding that GGA GAT binding to ubiquitin is enhanced by PI(4)P is very intriguing. Future work would be dedicated to more quantitative analysis of the interaction of GAT with ubiquitin in the presence of PI(4)P. We will use independent physiochemical and biochemical means to examine their interactions.

First of all, we are currently actively pursuing an assay based on surface plasmon resonance (SPR) (Morgan et al., 2001; Yu and Lemmon, 2001) in collaboration with Dr. Eileen Lafer at the University of Texas Health Science Center at San Antonio. Ubiquitin is immobilized on adjacent surfaces of a Biacore chip, and GAT recombinant protein, which is preincubated with PI(4)P liposome or solvent alone, passes over the two surfaces. Interaction of GAT-lipid with ubiquitin will be reflected as a time-dependent increase in the SPR response.

Recently Hurley and colleagues reported a measurement of GAT and ubiquitin interactions by isothermal titration calorimetry (ITC), a thermodynamic technique that allows the study of the interactions of two species in solution (Prag et al., 2005). ITC measures the binding equilibrium directly by determining the heat evolved on association of a ligand with its binding partner. To study the effect of PI(4)P on GAT and ubiquitin interaction, we plan to use the same procedure except incubating PI(4)P or solvent alone with GAT prior to its exposure to ubiquitin. We will analyze the data by comparing changes in the affinity of ubiquitin binding to GAT caused by PI(4)P versus solvent alone.

Interestingly, we found that both PI(4)P-binding mutants (GGA1R260A and GGA1R265A) are mislocalized to the plasma membrane (Figure 18C''). These cells also seem to have increased membrane ruffling. We have no definite explanation for these

phenomenons. However, competative binding of GGAs to Arf1 and Arf6 might be an answer. GGAs have been found to bind both Arf1 and Arf6 *in vitro* (unpulished data from Bonifacino lab), whereas *in vivo*, GGAs colocalize with Arf1 at the TGN, but not with Arf6 on the plasma membrane. This preference for TGN is presumably because Arf1 association is reinforced by other molecules at TGN, such as PI(4)P. GAT mutants that do not bind PI(4)P may therefore be recruited to the plasma membrane through binding to Arf6. To test this hypothesis, one simple way would be to overexpress Arf6 and examine the changes in cellular distribution of endogenous or overexpressed GGAs. It will also be interesting to see if cooverexpression of dominant negative Arf6 can eliminate the plasma membrane localization of the GGA PI(4)P-binding mutants.

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VITAE

Jing Wang was born in Shandong, China, on November 19, 1978, the only daughter of Yanping Yin and Fei Wang. After completing her studies at Jining High School, Shandong, China in 1996, Jing entered Shandong University at Jinan, Shandong. She received the degree of Bachelor of Science with a major in Microbiology from Shandong University in June, 2000. In August, 2000, she entered the Graduate School of Biological Sciences at the University of Missouri at Columbia. In January, 2001, Jing entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She began her dissertation research in the Integrative Biology Program under the supervision of Dr. Helen L. Yin since 2002. Jing and her husband Kun Yang were married in 2001. Their son, Max yi Yang, was born in 2002.

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