Canonical Interaction of Cyclin G-associated Kinase with Adaptor Protein 1 Regulates Lysosomal Enzyme Sorting

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The adaptor protein 1 (AP1) complex is a heterotetramer that participates in cargo sorting into clathrin-coated vesicles at the *trans*-Golgi network (TGN) and endosomes. The γ subunit of AP1 possesses a C-terminal “ear” domain that recruits a cohort of accessory proteins through recognition of a shared canonical motif, Ψ G[PDE][Ψ LM] (where Ψ is an aromatic residue). The physiological relevance of these ear-motif interactions, however, remains to be demonstrated. Here we report that the cyclin G-associated kinase (GAK) has two sequences fitting this motif, FGPL and FGEF, which mediate binding to the AP1- γ -ear domain in vitro. Mutation of both γ -ear-binding sequences or depletion of AP1- γ by RNA interference (RNAi) decreases the association of GAK with the TGN in vivo. Depletion of GAK by RNAi impairs the sorting of the acid hydrolase, cathepsin D, to lysosomes. Importantly, expression of RNAi-resistant GAK restores the lysosomal sorting of cathepsin D in cells depleted of endogenous GAK, whereas expression of a similar construct bearing mutations in both γ -ear-binding sequences fails to correct the sorting defect. Thus, interactions between the Ψ G[PDE][Ψ LM]-motif sequences in GAK and the AP1- γ -ear domain are critical for the recruitment of GAK to the TGN and the function of GAK in lysosomal enzyme sorting.

INTRODUCTION

Newly synthesized acid hydrolase precursors are modified with mannose 6-phosphate groups as they traverse the cisternae of the Golgi complex. This modification enables them to bind to transmembrane mannose 6-phosphate receptors (MPRs) upon arrival at the *trans*-Golgi network (TGN; Ghosh *et al.*, 2003). The resulting hydrolase-MPR complexes concentrate within clathrin-coated areas of the TGN by virtue of interactions between the cytosolic tails of the receptors and clathrin adaptors. This is followed by formation of clathrin-coated vesicles or carriers that transport the hydrolase-MPR complexes to endosomes. The low pH of the endosomal lumen dissociates these complexes, after which the hydrolase precursors are delivered to lysosomes while the receptors return to the TGN (Ghosh *et al.*, 2003). Two types of clathrin adaptor have been proposed to function in hydrolase-MPR complex sorting at the TGN (and possibly at endosomes as well): members of the GGA family of monomeric proteins and the adaptor protein 1 (AP1) heterotetrameric complex (Ghosh *et al.*, 2003). The GGA family comprises three members in mammals (GGA1, GGA2, and GGA3), each of which consists of four domains named VHS, GAT, hinge, and GAE (γ -adaptin ear) (Bonifacino, 2004).

AP1 is composed of four subunits, three of which occur as two or three distinct isoforms encoded by different genes. The subunits of AP1 and their isoforms (in parentheses) are named γ (γ 1 and γ 2), β 1, μ 1 (μ 1A and μ 1B), and σ 1 (σ 1A, σ 1B, and σ 1C) (Boehm and Bonifacino, 2002; Robinson 2004). The amino-terminal portions of γ and β 1, together with μ 1 and σ 1, form the “core” of AP1, whereas the carboxy-terminal portions of both γ and β 1 encompass two domains named “hinge” and “ear.”

The GAE domains of the GGA proteins and the ear domains of the AP1- γ subunit isoforms have a similar globular fold consisting of an eight-stranded immunoglobulin-like β -sandwich (Kent *et al.*, 2002; Nogi *et al.*, 2002; Collins *et al.*, 2003; Miller *et al.*, 2003). Consistent with this structural similarity, all five domains bind with various affinities to a common set of “accessory proteins,” including rabaptin-5 (Hirst *et al.*, 2000; Doray and Kornfeld, 2001; Shiba *et al.*, 2002; Mattera *et al.*, 2003), γ -synergizer (Page *et al.*, 1999; Hirst *et al.*, 2000; Takatsu *et al.*, 2000), p56 (Collins *et al.*, 2003; Lui *et al.*, 2003), NECA1 and NECA2 (Ritter *et al.*, 2003; Mattera *et al.*, 2004), aftiphilin (Mattera *et al.*, 2004), γ -BAR (Neubrand *et al.*, 2005), and a protein known as Clint, enthoproten, or epsinR (Kalthoff *et al.*, 2002; Wasiak *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003). Rabaptin-5 is part of a complex with the Rab5 guanine-nucleotide-exchange factor, rabex-5 (Horiuchi *et al.*, 1997; Mattera *et al.*, 2003), whereas γ -synergizer and aftiphilin form a complex with another protein named p200 (Hirst *et al.*, 2005). These accessory proteins share a canonical peptide motif that mediates interactions with the GGA-GAE and γ -ear domains (Duncan *et al.*, 2003; Mills *et al.*, 2003; Mattera *et al.*, 2003, 2004). Systematic bioinformatic, mutational, and binding analyses allowed us to define this canonical motif as Ψ G[PDE][Ψ LM] (where Ψ is an aromatic residue; pat-

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tern denoted according to PROSITE syntax; Mattera *et al.*, 2004; Table 1).

Peptides fitting this motif bind to two contiguous, shallow hydrophobic depressions formed by the side chains of basic groups on the ear domains (Collins *et al.*, 2003; Miller *et al.*, 2003; see Figure 2B). In addition, some GGA and AP1- γ accessory proteins bind directly to clathrin (e.g., enthopro-*tin*; Kalthoff *et al.*, 2002; Wasiak *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003), phosphoinositides (e.g., enthopro-*tin*; Kalthoff *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003), Rab proteins (e.g., rabaptin-5; Stenmark *et al.*, 1995; Vitale *et al.*, 1998), and NPF-motif-containing proteins (e.g., γ -synerg-*in*; Page *et al.*, 1999; Fernandez-Chacon *et al.*, 2000). Multiple protein interactions may thus contribute to the recruitment of GGA and AP1 accessory proteins to membranes. Despite the detailed understanding of the structural bases of Ψ G[PDE][Ψ LM]-ear interactions, their physiological relevance vis-à-vis other possible interactions remains to be demonstrated.

Here we report that the AP1-binding, cyclin G-associated kinase (GAK; also known as auxilin 2; Kanaoka *et al.*, 1997; Kimura *et al.*, 1997; Greener *et al.*, 2000; Umeda *et al.*, 2000; Lee *et al.*, 2005, 2006) specifically interacts with the ear domains of AP1- γ 1 and - γ 2 through two sequences fitting the Ψ G[PDE][Ψ LM] consensus motif. More importantly, we demonstrate that this interaction is required for the association of GAK with the TGN and for the transport of the precursor of the acid hydrolase, cathepsin D, to lysosomes. These findings constitute the first demonstration that a canonical interaction between AP1 and one of its accessory proteins is critical for lysosomal enzyme sorting.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM (Biosource, Rockville, MD) containing 4.5 g/l glucose, 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, and 10% vol/vol fetal bovine serum (FBS). Transient transfection of cells was performed using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturers' instructions.

Plasmids

The pEGFP-GAK and -auxilin plasmids were described previously (Lee *et al.*, 2006). Site-directed mutagenesis was carried out with the QuickChange kit (Stratagene, La Jolla, CA) using pEGFP-GAK as a template to introduce the F961A-L964A, F981A-F984A, and K69A substitutions. EcoRI-KpnI fragments of the wild-type (wt) and the double mutant GAK constructs were subcloned into the corresponding sites of the pPAGFP(A206K) plasmid to generate expression vectors for photoactivatable green fluorescent protein (PAGFP)-tagged GAK proteins. A mammalian expression vector encoding EGFP-AAK1 was kindly provided by Sean Conner (University of Minnesota, Minneapolis, MN). pGEX-mouse γ 1-ear was described previously (Mattera *et al.*, 2003). Site-directed mutagenesis of the γ 1-ear to introduce A753Q, K756Q, and R793Q substitutions was also performed using the QuickChange kit. For simplicity, enhanced GFP will hereafter be referred to as GFP.

Antibodies

Mouse monoclonal antibodies to AP1- γ 1, clathrin heavy chain (CHC), and GGA3 were purchased from BD Biosciences Pharmingen (San Diego, CA). Mouse monoclonal antibodies to GFP were purchased from Covance (Princeton, NJ) and Roche. Mouse mAb to CHC (clone X22) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Sheep polyclonal antibody to TGN46 was purchased from Serotec (Oxford, United Kingdom), and rabbit polyclonal antibody to cathepsin D was from Calbiochem (EMD Biosciences, San Diego, CA). A rabbit polyclonal antibody to GAK was described previously (Greener *et al.*, 2000). Donkey Alexa488- or Alexa594-conjugated anti-rabbit or anti-mouse IgG were purchased from Molecular Probes (Eugene, OR). Donkey Cy3-conjugated anti-sheep antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were purchased from Amersham Biosciences (Piscataway, NJ).

Pulldown Assays

HeLa cells were cultured on 10-cm dishes to 60–80% confluence. Twenty-four hours before the pulldown experiments, cells were transfected with the pEGFP-GAK, -AAK1 or -auxilin constructs. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% wt/vol Triton X-100, 1 $\times$ protease inhibitor cocktail [Roche], 1 $\times$ protein phosphatase inhibitor cocktail I and II [Sigma, St. Louis, MO]) for 30 min at 4°C, followed by centrifugation at 20,000 $\times$ g for 15 min. The supernatants were subsequently incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 30 min at 4°C, followed by centrifugation at 20,000 $\times$ g for 15 min, to yield precleared lysates. Thirty micrograms of purified GST or GST-fusion proteins was incubated with precleared lysates (derived from half a 10-cm dish) in the presence or absence of competing peptides for 2 h at 4°C with gentle mixing. The GST-protein complexes were subsequently pulled down by incubation with 30 μ l (bed volume) glutathione-Sepharose 4B beads for 1 h at 4°C. The beads were next washed three times with 1 ml ice-cold lysis buffer, and the bound proteins eluted by boiling in 40 μ l Laemmli buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

RNA Interference

RNA interference (RNAi) in HeLa cells was carried out as described previously (Janvier and Bonifacio, 2005). Small interfering RNA (siRNA) for CHC was also described previously (Janvier and Bonifacio, 2005). SmartPool siRNA for human AP1- γ 1 and a custom-designed siRNA for human GAK (5'-AACGAAGGAACAGCUGAUUCA-3') were purchased from Dharmacon, (Chicago, IL). This GAK siRNA targets the 3' untranslated region of the cDNA and, therefore, does not affect the expression of transfected GAK open reading frame (ORF) constructs lacking this region. Cells were analyzed after 72 h of treatment with siRNAs. In the rescue experiments, 48 h after transfection with GAK siRNA, the cells depleted of endogenous GAK were transfected with GFP-GAK ORF constructs using Lipofectamine 2000 and analyzed 24 h later. The transfection efficiency of these rescue plasmids was ~70% as assessed by fluorescent microscopy.

Isothermal Titration Calorimetry

Recombinant GST- γ 1-ear was expressed in *Escherichia coli* and purified with glutathione-Sepharose 4B according to the manufacturer's instructions. This protein as well as GAK peptides (Table 2; synthesized by EZBiolab, Westfield, IN) were dialyzed overnight at 4°C against excess isothermal titration calorimetry (ITC) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). All ITC experiments were carried out at 30°C using a VP-ITC instrument (MicroCal LLC, Northampton, MA). Typically, the chamber contained 1.4 ml of 40 μ M GST- γ 1-ear, and the GAK peptide (~1.4 mM) was added in 32 injections of 7 μ l each. Titration curves were analyzed using Origin software (MicroCal). Binding constants were calculated by fitting the curves corresponding to the wt and mutant GAK peptides to two- and one-site models, respectively (Table 3).

Fluorescence Microscopy and Image Analysis of Fixed Cells

Cells grown on cover glasses were fixed with 4% wt/vol paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 15 min at room temperature or with methanol:acetone (1:1) for 10 min at -20°C. Fixed cells were subsequently permeabilized for 5 min with 0.1% wt/vol Triton X-100 in PBS and blocked for 30 min with 1 mg/ml bovine serum albumin (BSA) at room temperature. After incubation with the primary and fluorescent secondary antibodies, the cells were imaged with a Zeiss 510 confocal microscope (Carl Zeiss, Thornwood, NY). For quantitative analysis of fluorescence intensities, nonsaturated images were captured with a Plan-Neofluar 25 $\times$ /0.80 objective and a fully open pinhole. Fluorescence quantification was performed as described previously (Kametaka *et al.*, 2005). In brief, intensities corresponding to total cellular fluorescence and TGN-associated fluorescence were measured using the NIH Image software after subtraction of background fluorescence outside the cells. Only cells that did not exhibit pixel saturation were used. Identical conditions were used for capturing the images corresponding to wt and mutant GFP-GAK constructs. Thirty-nine cells expressing wt GFP-GAK and 36 cells expressing the mutant GFP-GAK sites 1 and 2 (mut1/2) constructs were analyzed with the software, and the mean $\pm$ SD was calculated. The statistical significance of the differences in the distribution of wt and mutant GAK was analyzed using Student's *t* test.

Live Cell Imaging

Live cell imaging of GFP- or PAGFP-tagged GAK proteins were performed as described previously (Kametaka *et al.*, 2005) with slight modifications. In brief, HeLa cells transiently expressing GFP- or PAGFP-GAK fusion proteins grown on glass-bottom culture dishes (MatTek, Ashland, MA) were imaged in buffered medium using a Zeiss 510 confocal microscope equipped with a stage heated to 37°C. Selective photobleaching was performed using a 488-nm laser at full power, and recovery was monitored by time-lapse imaging at 5-s

Table 1. Accessory proteins that contain sequences fitting the Ψ G[PDE][Ψ LM] motif

Accessory protein	Sequence ^a
Rabaptin-5	436-ESD FGPL VG-444
γ -synergisin	455-EDD FQDF QE-463
γ -synergisin	667-ADD FGEF SL-675
Enthoprotin	341-ADL FGGF AD-349
Enthoprotin	369-NGD FGD WSA-377
p56	3-DDD FGGF EA-11
Aftiphilin	25-DDE FGEF GG-33
Aftiphilin	28-FGE FGGF SE-36
Aftiphilin	429-EDD FGDF GD-437
Aftiphilin	432-FGD FGDF GS-440
Aftiphilin	475-GDD FGEF GD-483
NECAP1	249-NDL WGDF ST-257
NECAP2	237-ADI WGDF TK-245
GAK	958-ADP FGPL L-966
GAK	978-PDL FGEF LN-986

^a The sequences fitting the Ψ G[PDE][Ψ LM] tetrapeptide motif are shown in bold. Underlined residues do not match the canonical motif. The numbering corresponds to the amino acid sequence of the human proteins.

intervals with low-intensity illumination. Photoactivation of PAGFP was carried out using a single hit from a 413-nm laser at full power, and fluorescence decay was monitored at 3-s intervals. Fluorescence quantification was carried out as described previously (Kametaka *et al.*, 2005). Each experiment was repeated at least five times under identical conditions, and the mean $\pm$ SD was graphically represented.

Metabolic Labeling and Pulse-Chase Analysis of Cathepsin D

Metabolic labeling of HeLa cells was carried out as described (Wasmeier *et al.*, 2005). Briefly, cells grown in a six-well plate were pulse-labeled for 2 h at 20°C using 0.1 mCi/ml [³⁵S]methionine-cysteine (Express Protein Label; Perkin Elmer-Cetus, Boston, MA). Cells were subsequently chased for 1–5 h at 37°C in regular culture medium in the presence of 5 mM mannose 6-phosphate and excess cold methionine (0.06 mg/ml) and cysteine (0.1 mg/ml). After chase, cells were rinsed twice with PBS and lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% (vol/vol) Triton X-100, supplemented with 1 $\times$ complete protease inhibitor cocktail (Roche). The extracts were immunoprecipitated with anti-cathepsin D antibody and analyzed by SDS-PAGE and fluorography. Quantification was performed using a Typhoon 9200 PhosphorImager (Amersham Biosciences) and ImageQuant analysis software.

In Vitro Kinase Assay

Lysates of HeLa cells transiently expressing GFP or GFP-GAK fusion constructs were subjected to immunoprecipitation with anti-GFP mAb. Equivalent amounts of immunoprecipitated GFP proteins were used for in vitro kinase assays using myelin basic protein (MBP) as a substrate (Rubinfeld and Seger, 1998). In brief, beads containing immunoprecipitated GFP proteins were incubated at 37°C for 30 min with 2 μ g MBP in 15 μ l of kinase reaction buffer (25 mM β -glycerophosphate, pH 7.3, 10 mM MgCl₂, 0.3% BSA, 1 mM dithiothreitol, 1 mM EGTA, 0.1 mM sodium orthovanadate, and 33 μ M [γ -³²P]ATP (~4000 cpm/pmol). After incubation, the kinase reaction was terminated by addition of 5 μ l 4 $\times$ Laemmli buffer and boiling for 5 min. Samples were analyzed by SDS-PAGE. The phosphorylated MBP and auto-phosphorylated GFP-GAK were detected on a phosphor imager.

RESULTS

GAK Contains Two Sequences Fitting the Ψ G[PDE][Ψ LM] Consensus Motif

A search of protein sequence databases using as a pattern the γ -ear- and GGA-GAE-binding motif, Ψ G[PDE][Ψ LM] (Mattera *et al.*, 2004), revealed two sequences fitting this motif, FGPL and FGEF, within a protein known as GAK or auxilin 2 (Kanaoka *et al.*, 1997; Kimura *et al.*, 1997; Table 1 and Figure 1). Both sequences are contained within a rela-

Figure 1. Schematic representation of GAK and related proteins. The different domains of auxilin, AAK1 and GAK/auxilin 2 are indicated. The scheme also shows the putative AP2-binding (DPF and WAAW) and clathrin-binding (DLL) sequences, as well as the two Ψ G[PDE][Ψ LM]-type, putative γ -ear-binding sequences (sites 1 and 2; highlighted in red) within the clathrin-binding (CB) domain of GAK.

tively unstructured segment (designated clathrin-binding or CB) of the protein that also contains binding motifs for the clathrin terminal domain (i.e., DLL) and for the ear domains of the AP2- α and - β 2 subunits (i.e., DPF and WAAW; Jha *et al.*, 2004; Mishra *et al.*, 2004; Figure 1). GAK also has a serine/threonine-kinase domain homologous to that of the mammalian adaptor-associated kinase AAK1 (Conner and Schmid, 2002) and the yeast Ark/Prk proteins (Zeng and Cai, 1999; Watson *et al.*, 2001), and tensin (also known as PTEN-homology) and J domains similar to those of auxilin (Ahle and Ungewickell, 1990; Figure 1). Previous studies showed that GAK is enriched in plasma membrane and TGN clathrin coats (Greener *et al.*, 2000; Lee *et al.*, 2006), where it phosphorylates the AP2- μ 2 and AP1- μ 1 subunits, respectively (Umeda *et al.*, 2000; Korolchuk and Banting, 2002). In addition, GAK was shown to promote clathrin uncoating, thus regulating receptor endocytosis at the plasma membrane (Greener *et al.*, 2000; Zhao *et al.*, 2001; Zhang *et al.*, 2004; Lee *et al.*, 2005) and sorting of MPRs and cathepsin D at the TGN (Lee *et al.*, 2005; Zhang *et al.*, 2005). These properties of GAK are consistent with the presence of putative γ -ear- and GGA-GAE-binding motifs within its sequence.

Preferential Binding of GAK to the Ear Domains of AP1- γ 1 and - γ 2

We performed glutathione S-transferase (GST) pulldown assays to examine the possible interaction of the ear domains of various clathrin adaptors with GAK and related proteins (Figure 2A). To this end, we expressed in HeLa cells GFP-tagged GAK, -auxilin or -AAK1. Lysates from these cells were tested for binding to immobilized GST-ear fusion proteins. In agreement with previous reports (Owen *et al.*, 1999; Umeda *et al.*, 2000; Conner and Schmid, 2002; Praefcke *et al.*, 2004), GAK, auxilin, and AAK1 were found to interact with the ear domain of the AP2- α C subunit isoform (Figure 2A). Importantly, GAK interacted with the ear domains of the AP1- γ 1 and - γ 2 subunit isoforms but not with the structurally related GGA-GAE domains (Figure 2A). In contrast, auxilin and AAK1 did not detectably bind to the γ 1- and γ 2-ear domains, and none of the tested proteins bound to the AP3- β 3B-ear domain (used here as a negative control; Figure 2A). As previously shown (Hirst *et al.*, 2000; Doray and Kornfeld, 2001; Shiba *et al.*, 2002; Mattera *et al.*, 2003), rabaptin-5

shown at the bottom of A and C. The apparent molecular masses of markers (in kDa) are indicated at the left of A and C.

interacted with the AP1-γ1- and -γ2-ear domains as well as the GAE domains of the three GGAs (positive control for GGA-GAE interactions), but not with the AP2 αC- and AP3 β3B-ear domains (Figure 2A). These experiments thus demonstrated that GAK binds specifically to the γ1- and γ2-ear domains.

GAK Binds to the Previously Defined Site for Accessory Proteins on the γ1-ear Domain

X-ray crystallographic and structure-based mutational analyses (Kent *et al.*, 2002; Nogi *et al.*, 2002) have identified the residues on the γ1-ear that are involved in interactions with sequences fitting the ΨG[PDE][ΨLM] motif (Figure 2B). GST pull-down assays showed that replacement of glutamine for either of two such γ1 residues, Lys756 and Arg793, substantially reduced binding to GAK (Figure 2C). A similar replacement for Ala753, which is less important for binding to other accessory proteins (Nogi *et al.*, 2002), had a weaker effect on GAK binding (Figure 2C). These results indicate that GAK binds to the previously defined site on γ1 for ΨG[PDE][ΨLM]-containing accessory proteins.

Binding to the γ1-ear Is Dependent on the GAK Sequences Fitting the ΨG[PDE][ΨLM] Motif

To assess whether binding to the γ1-ear is indeed dependent on the GAK FGPL and FGEF sequences, we carried out competition pull-down experiments using synthetic peptides encompassing the two tetrapeptide sequences (residues 958–988; Table 2). The wild-type GAK peptide (GAK wt) inhibited the interaction between GAK and the γ1-ear in a concentration-dependent manner (Figure 3A). Likewise, a wild-type enthoprotin peptide spanning residues 368–377 and containing a ΨG[PDE][ΨLM]-type sequence (FGDW; Table 2), inhibited the GAK–γ1-ear interaction with a potency similar to that of the GAK wt peptide (Figure 3A). Substitutions of key residues in both

the FGPL and FGEF sequences in the GAK peptide (mut1/2, Table 2) or in the FGDW sequence in the enthoprotin peptide (mut, Table 2), abrogated the ability of the peptides to compete (Figure 3B), indicating that the competition was mediated by the ΨG[PDE][ΨLM]-type sequences from both proteins. These observations demonstrated that the interaction with the γ1-ear is dependent on the ΨG[PDE][ΨLM] motifs in GAK.

Predominant Role of the FGEF Sequence in GAK for Interactions with the γ1-ear Domain

To discern the relative contribution of the FGPL and FGEF sequences in the binding of GAK to the γ1-ear, we performed competition pull-down assays using peptides with substitutions in each sequence (mut1 and mut2 peptides, respectively; Table 2). As shown in Figure 4A, although the mut1 peptide was almost as effective as the wt peptide in inhibiting binding of GAK to the γ1-ear, the mut2 peptide was inactive in this assay, indicating that the FGEF sequence is more important than the FGPL sequence for γ1-ear binding.

Table 2. Synthetic peptides used in this study

Peptide name	Sequence ^a
GAK wt	958–ADP FGPL LPSSGNNQPCSNPDL FGEF LNDS–988
GAK mut1	958–ADP AGPAL PSSGNNQPCSNPDL FGEF LNDS–988
GAK mut2	958–ADP FGPL LPSSGNNQPCSNPDL AGEA LNDS–988
GAK mut1/2	958–ADP AGPAL PSSGNNQPCSNPDL AGEA LNDS–988
Enthoprotin wt	368–CGNGD FGDWSA –377
Enthoprotin mut	368–CGNGD AGDSA –377

^a Sequences fitting the ΨG[PDE][ΨLM] motif are shown in bold and the substituted residues are underlined. Amino acid numbers are indicated.

Figure 3. Peptide competition of the interaction between GAK and the γ 1-ear. (A) Pull-down of GFP-GAK by immobilized GST- γ 1-ear was carried out as indicated in the legend to Figure 2 and in *Materials and Methods*, except that peptides derived from GAK or enthoprotin (wt sequences; Table 2) were added to the incubation mixture at concentrations ranging from 75 to 0.3 μ M in 1:3 serial dilutions. Samples corresponding to 5% of the input extract and the pull-down of GFP-GAK by GST- γ 1-ear in the absence of peptide (–) were run on the first two lanes for comparison. (B) Pull-down of GFP-GAK by immobilized GST- γ 1-ear in the absence (–) or presence of 75 μ M of the wild-type (wt) or mutant GAK (mut1/2) peptides, or the wild-type (wt) or mutant (mut) enthoprotin peptides described in Table 2. In both A and B, bound GFP-GAK was detected by immunoblotting (IB) with antibody to GFP. Blots were also probed for GGA3 as a specificity control. Ponceau Red staining of blotted proteins is shown at the bottom of both panels. The apparent molecular masses of markers (in kDa) are indicated on the left of both panels.

We also mutated the FGPL and/or FGEF sequences in the context of full-length GFP-GAK and performed pull-down assays with GST-fusions to the γ 1-ear, α C-ear, or GGA3-GAE domains (the latter two as positive and negative controls, respectively; Figure 4B). Consistent with the peptide competition experiments (Figure 4A), mutation of FGEF substantially reduced binding to the γ 1-ear (mut2, Figure

Figure 4. Relative contributions of two canonical GAK sequences to binding to the γ 1-ear. (A) Wild-type GAK peptide (wt) and GAK peptides with substitutions in two Ψ G[PDE][Ψ LM]-motif sequences (mut1, mut2, or mut1/2; see Table 2; used at 75 μ M) were examined for their ability to inhibit the GAK- γ 1-ear interaction in GST pull-down assays similar to those described in the legend to Figure 3 and in *Materials and Methods*. (B) Extracts of HeLa cells expressing GFP-tagged, full-length GAK (wt or substituted as in the mut1, mut2, or mut1/2 peptides; Table 2) were used in pull-down assays with GST- γ 1-ear, - α C-ear, and -GGA3-GAE as described in the legend to Figure 2 and in *Materials and Methods*. Ponceau Red staining of blotted proteins is shown at the bottom of A and B. The apparent molecular masses of markers (in kDa) are indicated on the left of both panels.

4B). Mutation of FGPL alone, on the other hand, had no effect on binding (mut1, Figure 4B), but had a synergistic effect when combined with the FGEF mutation (mut1/2, Figure 4B). None of these mutations affected the interaction of GAK with the α C-ear, underscoring the specificity of the interaction with the γ 1-ear.

Finally, we analyzed these interactions by ITC using recombinant GST- γ 1-ear and synthetic GAK peptides (Table 2) as ligands. We found that the wt GAT peptide

Figure 5. ITC analysis of the binding of GAK peptides (wt, mut1, mut2, and mut1/2; Table 2) to GST- γ 1-ear, performed as described in *Materials and Methods*. The binding isotherms shown are representative of at least three experiments per peptide. The K_d values estimated from these experiments are shown in Table 3. The inset shows the heat change elicited by serial injections of wild-type GAK peptide onto GST- γ 1-ear.

Table 3. Binding affinities of GAK peptides for GST- γ 1-ear calculated from ITC data

GAK peptide	K_d (μ M)
Wild-type ^a	47; 1200
FGPL mutant (mut1) ^b	228
FGEF mutant (mut2) ^b	~2700
FGPL-FGEF double mutant (mut1/2) ^b	ND

Equilibrium dissociation constants (K_d) values calculated after fitting the titration data from Figure 5 to two-site^a or single-site^b models. ND, K_d was not determined because binding was too weak to obtain reliable quantification.

bound to the γ 1-ear (Figure 5) with an isotherm that could be best fitted to a two-site model, yielding equilibrium dissociation constants of 47 and 1200 μ M for each site (Table 3). Amino acid substitutions in each motif yielded single-site isotherms with very different effects on affinity. Although substitutions in the FGPL motif (mut1) alone had only a slight effect on the interaction, substitutions in the FGEF motif alone (mut2) or in both motifs together (mut1/2) largely abrogated the interaction (Figure 5 and Table 3). Taken together, these experiments indicated that interactions with the γ 1-ear are mainly mediated by the FGEF sequence in GAK.

Association of GAK with the TGN Is Dependent on AP1

We next examined whether the interactions described above are important for the intracellular localization of GAK. In

Figure 6. Colocalization of GAK with TGN46, clathrin and AP1 at the TGN, and effect of brefeldin A. HeLa cells incubated in the absence (A–I) or presence of 5 μ g/ml brefeldin A for 15 min (J–L) were fluorescently coimmunostained for endogenous GAK (A, D, G, and J) and TGN46 (B), clathrin heavy chain (E), or AP1- γ 1 (H and K), and examined by confocal microscopy as described in *Materials and Methods*. C, F, I, and L are merged images. Bars, 10 μ m.

agreement with previous reports (Greener *et al.*, 2000; Zhang *et al.*, 2005; Lee *et al.*, 2006), immunofluorescence microscopy analyses showed that a fraction of endogenous GAK colocalized with TGN46 (Figure 6, A–C), clathrin (Figure 6, D–F), and AP1 (Figure 6, G–I) at the TGN. Treatment with brefeldin A (BFA), which dissociates Arf1-regulated coat proteins such as AP1 from membranes (Robinson and Kreis, 1992; Wong and Brodsky, 1992), redistributed GAK to the cytosol (Figure 6, J–L). Treatment for 3 d with specific siRNAs showed that depletion of the γ 1 subunit of AP1 (Figure 7A) caused dissociation of GAK from the TGN (Figure 7, E–G). Depletion of GAK, on the other hand, did not lead to dissociation of AP1 from membranes, although it did cause some dispersal of AP1-containing structures (Figure 7, B–D). These changes were not due to disruption of the Golgi complex, because the distribution of the Golgi-stack marker GM130 was unaffected by depletion of AP1 subunits or GAK (data not shown). These results thus demonstrated that association of GAK with the TGN is dependent on AP1. Longer periods of siRNA treatment (i.e., 5–7 d) did cause disruption of the Golgi complex in some cells, as detected by staining with antibodies to GM130 and TGN46 (data not shown). This was in agreement with previous observations made by plasmid-based RNAi knockdown, in which the cells were selected with hygromycin B for 5–7 d after transfection with RNAi vectors (Lee *et al.*, 2005). Golgi disruption upon prolonged RNAi treatment may be due to more complete depletion of GAK or to the accumulated effects of impaired trafficking. To avoid this effect on Golgi structure,

Figure 7. AP1- $\gamma 1$ depletion decreases association of GAK with the TGN. (A) Levels of endogenous GAK, AP1- $\gamma 1$, and actin in cells from control and GAK-RNAi- and AP1- $\gamma 1$ -RNAi-treated HeLa cells. (B–G) Immunofluorescence microscopy for AP1- $\gamma 1$ (B–D) and GAK (E–G) in control HeLa cells (B and E) and HeLa cells treated with siRNAs for GAK (C and F) or AP1- $\gamma 1$ (D and G). Asterisks indicate a cell in which AP1- $\gamma 1$ expression was not silenced. Bar, 10 μ m.

subsequent functional experiments (i.e., see Figures 10 and 11) were performed by depleting cells of GAK for 3 d.

The γ -ear-binding Motifs Contribute to the Association of GAK with the TGN

To determine the importance of the γ -ear-binding motifs for the association of GAK with the TGN, we compared the localization of GFP-tagged forms of wild-type GAK (wt) and GAK with mutations in both the FGPL and FGEF sequences (mut1/2) in transiently transfected HeLa cells. GFP-tagged wt GAK showed good localization to the TGN, as determined by costaining with antibody to TGN46 (Figure 8, A–F). Mutation of the FGPL and FGEF sequences, however, caused a shift in the distribution of GAK from the TGN to the cytosol, although a small amount of TGN staining could be seen in some cells (Figure 8, G–I; quantitative analysis shown in Figure 8, J and K). Fluorescence recovery after photobleaching (FRAP) analysis of the TGN showed a faster rate of recovery for mut1/2 GAK relative to wt GAK (Figure 9A, Table 4). Moreover, the dissociation of PAGFP-GAK mut1/2 from the TGN was also faster than that of GAK wt (Figure 9B, Table 4). These results indicate that mutation of the FGPL and FGEF motifs results in faster exchange of GAK between TGN and cytosolic pools, which reflects a looser association of mutant GAK with TGN clathrin/AP1 coats.

Binding of GAK to AP1 Is Required for its Function in Lysosomal Enzyme Sorting

Depletion of GAK has been recently shown to affect lysosomal sorting both in altering the localization of MPRs (Lee *et*

Figure 8. The ability of GAK to bind AP1 is important for its association with the TGN. HeLa cells transiently transfected with plasmids encoding wt (A–F) or mut1/2 (G–I) GFP-GAK were fluorescently counterstained with antibody to TGN46 (B, E, and H). A–C and D–F are two examples of the colocalization of GFP-GAK with TGN46 at different magnification. Bars, 10 μ m. (J) Quantitative analysis of the relative fluorescence intensity of wt ($\circ$; 39 cells) and mut1/2 ($\bullet$; 36 cells) GFP-GAK proteins associated with the TGN. (K) Mean $\pm$ SD of datasets in G are represented by bar graphs. The statistical significance of the difference in TGN association was assessed by Student's *t* test (**p* < 0.001).

et al., 2005) and delaying the maturation of cathepsin D (Zhang *et al.*, 2005). This maturation involves successive proteolytic cleavage from precursor (pro-catD) to intermediate (int-catD) and, eventually, to mature cathepsin D (m-catD). Immunoblot analysis showed that depletion of GAK or clathrin increased the proportion of pro-catD and int-catD, while decreasing the proportion of m-catD, at steady state (Figure 10A). Consistent with these results, pulse-chase analysis showed that depletion of GAK or clathrin for 3 d delayed the processing of pro-catD to int-catD and then to m-catD (Figure 10, B and C). Importantly, transfection with RNAi-resistant GFP-GAK (wt) corrected the cathepsin D maturation defect in GAK-depleted cells, whereas RNAi-resistant GFP-GAK with mutations in the FGPL and FGEF sequences (mut1/2) or kinase-deficient GAK K69A (Zhang *et al.*, 2005; Figure 11A) did not rescue cathepsin D maturation, as determined by immunoblot analysis (Figure 11B). These results indicated that both the ability of GAK to interact with AP1 and its kinase activity are important for the role of GAK in the lysosomal sorting of cathepsin D.

Figure 9. Dynamics of GAK association with the TGN in live cells analyzed by photobleaching and photoactivation. (A) The kinetics of wt and mut1/2 GFP-GAK recruitment to the TGN in transiently transfected HeLa cells were analyzed by fluorescence recovery after photobleaching (FRAP) as described in *Materials and Methods* and in a previous publication (Kametaka *et al.*, 2005). (B) The kinetics of dissociation from the TGN of wt and mut1/2 GAK tagged with photoactivatable GFP (PAGFP) in transiently transfected HeLa cells were analyzed after photoactivation of the TGN-associated population of PAGFP-GAK as described in *Materials and Methods*. Data points represent the mean $\pm$ SD from five experiments.

Table 4. Kinetics of membrane association and dissociation of GFP-GAK fusion proteins

Method	GFP-GAK	$t_{1/2}$ (s)	Initial rate (%/s)
Fluorescence recovery after photobleaching	wt	17.2 ± 7.6	4.2 ± 4.1
	mut1/2	4.3 ± 4.5	11.1 ± 4.9
Dissociation after photoactivation	wt	10.2 ± 2.5	5.1 ± 2.1
	mut1/2	4.4 ± 2.1	7.8 ± 2.3

Calculations were made from the data shown in Figure 9.

DISCUSSION

The results presented here add GAK to the growing list of accessory proteins that interact through canonical motifs with the ear domain of the γ subunit of AP1. The ability of GAK to interact in this manner with AP1 is critical for its recruitment to the TGN (Figures 7–9) and for lysosomal enzyme sorting (Figure 11). To our knowledge, these findings represent the first demonstration that canonical motif-ear interactions are functionally relevant for protein sorting in vivo.

Interaction of GAK with the Ear Domains of AP1- γ 1 and - γ 2

GAK has two contiguous sequences, FGPL (residues 961–964) and FGEF (residues 981–984), that fit the consensus motif, Ψ G[PDE][Ψ LM], for γ -ear binding (Mattera *et al.*, 2004). Both sequences are preceded by acidic residues (Asp at -2 relative to the Phe defined as position 0), which are typical of known γ -ear-binding sequences (Duncan *et al.*, 2003; Mattera *et al.*, 2003, 2004; Mills *et al.*, 2003). Moreover, both sequences occur within a segment of GAK that is predicted to be largely unstructured and therefore accessible for interactions with γ -ear domains. Our experimental analyses bear out these predictions by demonstrating that, indeed, both the FGPL and FGEF sequences mediate binding of GAK to γ -ear domains in vitro (Figures 2–5). The FGPL sequence does so with lower affinity probably due to the proline residue at $+2$, which might hinder the ability of the tetrapeptide to adopt a favorable conformation for binding. Despite their marked differences in affinity, both sequences contribute to the overall binding avidity of full-length GAK (Figure 4B).

A noteworthy feature of these interactions is the preference of GAK for the ear domains of the γ 1 and γ 2 subunit isoforms of AP1 over the GAE domains of the three GGA proteins (Figure 2). Other accessory proteins such as γ -syn-ergin, enthoprotin, and NECAP1, which share sequences fitting the Ψ G[PDE][Ψ LM] motif (Table 1), exhibit a similar preference for γ -ears (Mills *et al.*, 2003; Mattera *et al.*, 2004; Ritter *et al.*, 2004). This is in contrast to p56 (Collins *et al.*, 2003; Lui *et al.*, 2003) and rabaptin-5 (Mattera *et al.*, 2003), which bind to γ -ear and GGA-GAE domains with comparable avidities. These distinct patterns of interaction are intriguing because γ -ear and GGA-GAE domains share a similar fold and a conserved motif-binding site (Kent *et al.*, 2002; Nogi *et al.*, 2002; Miller *et al.*, 2003). The selectivity of binding must thus be due to the exact identity of residues within each motif and to subtle differences in the properties of the motif-binding site.

Recruitment of GAK to the TGN Requires Interaction with AP1- γ

Consistent with the direct interaction of GAK with AP1 demonstrated here, these two proteins colocalize to a jux-tanuclear region of the cytoplasm that contains the TGN and a subset of endosomes (Greener *et al.*, 2000; Umeda *et al.*, 2000; Lee *et al.*, 2006; Figure 6). For simplicity, we refer to this localization as TGN, although our observations are equally applicable to endosomes, where a population of AP1 is known to be located (Futter *et al.*, 1998). Using RNAi-mediated silencing, we found that depletion of AP1 causes a substantial decrease in the association of endogenous GAK with the TGN (Figure 7). Moreover, a transgenic GAK construct with substitutions in both the γ -ear binding FGPL and FGEF motifs shows reduced TGN localization at steady state (Figure 8) and faster exchange between the TGN and the cytosol (Figure 9). These observations indicate that the ability of GAK to interact with AP1 is critical for its localization to the TGN.

Notwithstanding the predominant role of GAK-AP1 interactions, a small amount of GAK associates with the TGN independently of its interaction with AP1 (Figures 8 and 9). This suggests that additional interactions might contribute to GAK recruitment to the TGN. GAK has clathrin-binding motifs (Figure 1), but RNAi-mediated depletion of clathrin had no discernible effect on GAK localization to the TGN (our unpublished observations). A more likely contributor to

Figure 10. The GAK-AP1 interaction is important for lysosomal enzyme sorting. (A) Immunoblot (IB) analysis of pro- (pro-catD), intermediate (int-catD), and mature cathepsin D (m-catD) in untreated HeLa cells (control) and HeLa cells treated with siRNAs directed to GAK or clathrin heavy chain (CHC). Cell lysates were resolved by SDS-PAGE followed by immunoblotting with antibodies to cathepsin D, GAK, and CHC. (B) Pulse-chase analysis of cathepsin D maturation in [³⁵S]methionine-cysteine-labeled HeLa cells treated with control, GAK, and CHC siRNAs as described in *Materials and Methods*. (C) Densitometric quantification of the results from B for the different cathepsin D species. The percentage of each species at each time point was normalized by the corresponding number of methionine and cysteine residues.

TGN localization is the tensin/PTEN-homology domain (Figure 1), which binds various phosphoinositides and regulates the dynamics of GAK association with clathrin-coated pits at the plasma membrane (Lee *et al.*, 2006).

Requirement of the GAK-AP1 Interaction for Lysosomal Enzyme Sorting

Both AP1 (Meyer *et al.*, 2000; Hirst *et al.*, 2003, 2005) and GAK (Zhang *et al.*, 2005) are required for the sorting of the precursor of the acidic hydrolase, cathepsin D, to lysosomes. Using an RNAi-rescue approach, we showed that the ability of GAK to bind to AP1 is critical for the lysosomal sorting of cathepsin D (Figure 11). Thus, even a catalytically active GAK cannot function in lysosomal enzyme sorting unless it is recruited to the TGN by virtue of its canonical motif-ear interaction with AP1. These findings suggest that similar canonical interactions with AP1 might contribute to the recruitment of other accessory proteins to the TGN and to their possible function in protein sorting. Because AP1 and GAK are both components of TGN clathrin coats, it is not surprising that RNAi-mediated depletion of clathrin also causes missorting of cathepsin D (Figure 10). This latter finding is in agreement with observations from a previous study in which clathrin expression was reduced by antisense RNA (Iversen *et al.*, 2003).

The involvement of AP1 in the sorting of cathepsin D could depend on its role in the packaging of mannose 6-phosphate receptors into clathrin-coated vesicles at the TGN (Doray *et al.*, 2002). However, AP1 has also been proposed to be localized to endosomes (Futter *et al.*, 1998) and

to play a role in the sorting of mannose 6-phosphate receptors from endosomes to the TGN (Meyer *et al.*, 2000). These findings raise the possibility that AP1 could mediate bidirectional transport between the TGN and endosomes. GAK could thus play its role in lysosomal enzyme sorting through interaction with AP1 at the TGN, endosomes, or both.

The effects of GAK depletion on cathepsin D sorting were examined in cells treated for 3 d with synthetic siRNAs, which caused 80–90% decrease in the levels of endogenous GAK. Under these conditions, cathepsin D was missorted even though the Golgi/TGN remained intact. Thus, these early effects were likely directly related to decreased GAK function. Longer treatments may lead to a greater decrease of endogenous GAK but result in varying degrees of Golgi/TGN disruption, as previously reported (Lee *et al.*, 2005). Prolonged or more complete GAK depletion therefore leads to drastic alteration of the structure of the Golgi complex, which could be an indirect effect of GAK loss.

How Does GAK Participate in Lysosomal Enzyme Sorting?

Although it is now clear that both the expression of GAK (Lee *et al.*, 2005; Zhang *et al.*, 2005; this study) and its ability to interact with AP1 (this study) are required for lysosomal enzyme sorting, the exact mechanism by which GAK participates in this process remains to be elucidated. GAK contains a Ser/Thr kinase domain homologous to that of AAK1, an enzyme that phosphorylates the $\mu 2$ subunit of AP2 and activates it for recognition of YXX Φ -type sorting signals (Conner and Schmid, 2002; Ricotta *et al.*, 2002; Honing *et al.*,

Figure 11. Both the AP1- γ 1-ear-binding site and kinase activity of GAK are required to reverse the defect in cathepsin D maturation in GAK-depleted cells. (A) In vitro kinase assay using GFP alone and GFP fused to wild-type (wt), AP1- γ 1-ear-binding site mutant (mut1/2) or kinase-deficient (K69A; Zhang *et al.*, 2005) GAK. GFP proteins were expressed by transfection in HeLa cells, immunoprecipitated with anti-GFP antibody, and analyzed using an in vitro kinase assay with myelin basic protein (MBP) as a substrate, as described in *Materials and Methods*. Autophosphorylated GFP-GAK proteins (asterisk) and phosphorylated MBP labeled with 32 P are indicated in the left panel; the amounts of GFP proteins used for the assay, as detected by immunoblotting with anti-GFP antibody, are shown in the right panel. Notice that mutation of the AP1- γ 1-ear-binding site has no effect on kinase activity, whereas mutation of the active site K69 residue decreases GAK autophosphorylation and completely abrogates MBP phosphorylation. (B) HeLa cells depleted of GAK by treatment with siRNA to the 3' UTR were transfected with either wt, mut1/2, or K69A GFP-GAK ORF cDNAs, as described in *Materials and Methods*, and analyzed by SDS-PAGE and immunoblotting with antibody to cathepsin D (top panel; cathepsin D species are denoted as indicated in the legend to Figure 10). Expression of the GFP-GAK constructs was determined by immunoblotting with antibody to GFP (bottom panel). Notice that both mut1/2 and K69A GAK fail to rescue cathepsin D processing in GAK-depleted cells. Moreover, K69A GAK appears to exert a dominant-negative effect, because it causes a buildup of pro-catD. The apparent molecular masses of markers (in kDa) are indicated on the left.

2005). Indeed, GAK has been shown to phosphorylate the μ 1 subunit of AP1 in vitro (Umeda *et al.*, 2000; however, see also Korolchuk and Banting, 2002). By analogy to μ 2, GAK-catalyzed phosphorylation of μ 1 could activate AP1 for recognition of some sorting signal in the tails of the mannose 6-phosphate receptors (Ghosh *et al.*, 2003; Ghosh and Kornfeld, 2004). The kinase domains of both GAK and AAK1 are homologous to those of *Saccharomyces cerevisiae* Ark/Prk proteins. These yeast proteins have been shown to regulate

endocytosis and the actin cytoskeleton through phosphorylation of multiple substrates (Smythe and Ayscough, 2003). Thus, the role of GAK in lysosomal enzyme sorting could involve phosphorylation of other components of the trafficking machinery or the actin cytoskeleton. Although the key GAK substrate involved in the biosynthetic sorting of cathepsin D remains to be identified, we have shown that that kinase activity of GAK is essential for this process (Figure 11). GAK also contains a J domain homologous to that of auxilin (Greener *et al.*, 2000). This domain cooperates with the chaperone Hsc70 to uncoat clathrin-coated vesicles (Greener *et al.*, 2000; Umeda *et al.*, 2000). It is therefore also possible that GAK functions to remove the coat from TGN- or endosome-derived clathrin-coated vesicles that transport mannose 6-phosphate receptors. The interaction of GAK with AP1 described here thus likely enables the recruitment of both the kinase and uncoating activity of GAK to sites of lysosomal enzyme sorting.

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