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The phospholipase complex PAFAH Ib regulates the functional organization of the Golgi complex

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We report that platelet-activating factor acetylhydrolase (PAFAH) Ib, comprised of two phospholipase A2 (PLA2) subunits, α1 and α2, and a third subunit, the dynein regulator lissencephaly 1 (LIS1), mediates the structure and function of the Golgi complex. Both α1 and α2 partially localize on Golgi membranes, and purified catalytically active, but not inactive α1 and α2 induce Golgi membrane tubule formation in a reconstitution system. Overexpression of wild-type or mutant α1 or α2 revealed that both PLA2 activity and LIS1 are important for maintaining Golgi structure. Knockdown of PAFAH Ib subunits fragments the Golgi complex, inhibits tubule-mediated reassembly of intact Golgi ribbons, and slows secretion of cargo. Our results demonstrate a cooperative interplay between the PLA2 activity of α1 and α2 with LIS1 to facilitate the functional organization of the Golgi complex, thereby suggesting a model that links phospholipid remodeling and membrane tubulation to dynein-dependent transport.

Introduction

An unresolved feature of the Golgi complex is the role of membrane tubules in trafficking and maintenance of Golgi architecture (Bard and Malhotra, 2006; Pfeffer, 2007; De Matteis and Luini, 2008). Within the Golgi, membrane tubules are implicated in cis to trans cargo transport (Marsh et al., 2004; Trucco et al., 2004), retrograde trafficking to the ER (Lippincott-Schwartz et al., 1989; Saraste and Svensson, 1991), transport from the TGN to the cell surface and endosomes (Puertollano et al., 2001; Waguri et al., 2003; Bard and Malhotra, 2006; De Matteis and Luini, 2008), and in the assembly and maintenance of intact Golgi ribbons (de Figueiredo et al., 1998, 1999).

Pharmacological studies have shown cytoplasmic phospholipase A2 (PLA2) enzymes regulate the formation of Golgi membrane tubules that contribute to retrograde trafficking, and the assembly and maintenance of an intact Golgi ribbon in mammalian cells (de Figueiredo et al., 1998, 1999, 2000; Drecktrah and Brown, 1999; Polizotto et al., 1999; Brown et al., 2003). Here we identify platelet-activating factor acetylhydrodrolase (PAFAH) Ib as a cytoplasmic PLA2 complex that regulates membrane tubule formation and organization and function of the Golgi complex. PAFAH Ib was originally purified based on its ability to hydrolyze the sn-2 position acetyl group of the signal-transducing, phosphatidylcholine derivative, platelet-activating factor (PAF; Hattori et al., 1993, 1994a,b). PAFAH Ib consists of homo- or hetero-dimers of two closely related catalytic subunits, α1 (Pafah1b3) and α2 (Pafah1b2), and a non-catalytic dimer of β subunits (Pafah1b1; Ho et al., 1997; Arai et al., 2002; Tarricone et al., 2004). The β subunit is better known as LIS1, the causative agent of the fatal brain disorder Miller-Dieker lissencephaly (Kato and Dobyns, 2003). LIS1 is a highly conserved protein involved in dynein-mediated processes including nuclear and neuronal migration, centrosomal function, and mitotic spindle orientation (Kerjan and Gleeson, 2007; Vallee and Tsai, 2006). Although PAFAH Ib has been implicated in a variety of processes, including the regulation of PAF and LIS1 functions, its exact biological function is unclear, as α1−/−/α2−/− double-knockout mice, although exhibiting defects...
Other proteins identified in the GF fraction were Hsc70, PLAA-activating protein (PLAA), and fructose bisphosphate aldolase. A variety of experiments showed that aldolase is a contaminant; the role of Hsc70 and PLAA in tubule formation, if any, remains to be determined.

To determine if PAFAH Ib is directly involved in membrane tubule formation, purified $\alpha_1$ or $\alpha_2$ was added to isolated Golgi complexes in vitro. Alpha subunits alone were unable to stimulate membrane tubules in the absence of BBC (Fig. 1 G). However, when $\alpha_1$ or $\alpha_2$ was mixed with subthreshold amounts of BBC, catalytically active $\alpha_1$ and $\alpha_2$ but not inactive enzymes with single amino acid changes in active site serines ($\alpha_1S47A$; $\alpha_2S48A$; Hattori et al., 1994b) significantly induced Golgi membrane tubule formation (Fig. 1, C–E). This cytosol requirement was not due to LIS1, because $\alpha_1$ or $\alpha_2$ mutants that do not bind to LIS1 ($\alpha_1E38D$ and $\alpha_2E39D$; Yamaguchi et al., 2007) stimulated membrane tubules (Fig. 1 G). Consistent with these results, antibodies against $\alpha_1$ and $\alpha_2$ (but not preimmune antisera) inhibited BBC-dependent in vitro Golgi membrane tubulation (Fig. 1 F), whereas the addition of LIS1 antibodies had no effect (unpublished data). These results show that PAFAH Ib catalytic activity is required for stimulating membrane tubules from isolated Golgi complexes.

in spermatogenesis, are otherwise normal (Koizumi et al., 2003; Yan et al., 2003).

Using multiple in vitro and in vivo approaches, we find that all three subunits of PAFAH Ib contribute to the structure of the mammalian Golgi complex, and the catalytic subunits independently contribute to secretion. Our results suggest a model whereby the PLA$_2$ activity of $\alpha_1$ and $\alpha_2$ remodel membrane phospholipids to form Golgi tubules, which are linked via LIS1 to dynein-mediated, microtubule transport.

Results and discussion

**PAFAH Ib $\alpha_1$ and $\alpha_2$ are cytoplasmic membrane tubulation factors**

Previous studies showed that a preparation of bovine brain cytosol (BBC) contains PLA$_2$ activity that stimulates Golgi membrane tubule formation in a cell-free system (de Figueiredo et al., 1999). Biochemical fractionation of BBC yielded a final Golgi tubule–stimulating activity (determined by an in vitro reconstitution assay) cofractionates with PAFAH activity and PAFAH Ib subunits (Western blot) in final bovine brain cytosol (BBC) fractionation steps. (B) Silver stain of final fractions with the minimal components that stimulate Golgi tubules in vitro. The components identified by MALDI TOF-TOF and/or Western blot are labeled (NS = non specific). (C) Coomassie-stained gel and PAFAH activity of purified subunits. (D) EM of negative stain Golgi from in vitro tubulation reconstitution assays incubated with BBC and purified subunits as indicated. Bars, 500 nm. (E) Quantitation of tubulation assay using purified $\alpha_1$ and $\alpha_2$ subunits (7.5 $\mu$g/ml), with subthreshold BBC (low BBC, 0.25 mg/ml) compared with saturating BBC (1.5 mg/ml). svPLA$_2$ = snake venom PLA$_2$.

Results are the percentage of control (BBC), $n = 4$; error bars = SD. (F) Anti-$\alpha_1$ and/or anti-$\alpha_2$ antibodies, but not preimmune IgG (PI-$\alpha_1$, PI-$\alpha_2$), inhibit cytosol-stimulated Golgi membrane tubules in vitro. BSA = bovine serum albumin. Results are the percentage of control (BBC), $n = 4$; error bars = SD. (G) Quantitation of tubulation with purified wild-type or LIS1-binding defective $\alpha_1E38D$ and $\alpha_2E39D$ (7.5 $\mu$g/ml), in the presence or absence of low BBC. Results are percentage of control (BBC), $n = 3$; error bars = SEM.
PAFAH Ib α1 and α2 partially localize to Golgi membranes and tubules

In mildly expressing cells, catalytically active HA-tagged α1 or α2 (Fig. 1 C) was found diffuse throughout the cytoplasm, in the nucleus, on punctate structures, and clearly on intracellular structures in the juxtanuclear region that colocalized with Golgi markers including Rab6-GFP (cis), GPP130 (cis), mannosidase II (ManII, medial), and mannose 6-phosphate receptors (M6PR, TGN; Fig. 2, A and B; Fig. S1 A; unpublished data). A similar distribution was seen by Caspi et al. (2003) in cells expressing GFP-α1. Catalytically inactive α1 S47A and α2 S48A, as well as α1 E38D and α2 E39D, were similarly localized (Fig. S1 A; unpublished data), indicating that catalytic activity and LIS1 binding are not required for α1 or α2 association with Golgi membranes. In addition to centrosomal and cytoplasmic localization, HA-tagged LIS1 was observed to surround the Golgi complex (Fig. 2, D and F), consistent with reports that a small fraction of LIS1 is membrane associated (Lam et al., 2010). PAAFH Ib α1 could also be found localized to membrane tubules (Fig. 2, E and G; Fig. S1 B). Consistent with the imaging results, we found that a fraction of both α1 and LIS1 colocalized with Golgi membranes in sucrose gradients (Fig. 2 C).

PAFAH Ib α1 and α2 overexpression reveals a connection between PLA2 activity and LIS1-mediated regulation of Golgi structure

Overexpression of α1 or α2 resulted in a fragmented or completely dispersed Golgi and TGN (Fig. 2, H and I; Fig. S2), which was more severe in α2-overexpressing cells (Fig. S2, A, C, and D). Changes in Golgi structure were not observed when cells overexpressed an unrelated cytoplasmic PLA2, iPLA2-α (unpublished data). Overexpression of catalytically inactive α1 S47A or α2 S48A also caused the Golgi and TGN to become fragmented or diffuse (Fig. 2, H and I; Fig. S2). Overexpression of catalytically inactive α1 or α2 may produce a dominant-negative effect by forming poisonous dimers, competing with endogenous PLA2 subunits also competing for binding to membranes, and/or by titrating LIS1, thus inhibiting the ability of LIS1 to regulate dynein (Vallee and Tsai, 2006; Kerjan and Gleeson, 2007; Ding et al., 2009).

Microtubules and dynein-mediated centripetal positioning are required for the maintenance of an intact mammalian Golgi near the centrosome (Corthésy-Theulaz et al., 1992). To determine if overexpression of α1 and α2 affected Golgi structure by binding to and influencing LIS1 function, cells were transfected with the LIS1-binding mutants α1 E38D or α2 E39D. α2 E39D did disrupt the Golgi and TGN, albeit less severely than α2 wild-type or α2 S48A (Fig. 2 I; Fig. S2, D and E). In contrast to wild-type and catalytically inactive α1, overexpression of equivalent levels of α1 E38D did not significantly disrupt the Golgi or TGN (Fig. 2, H and I; Fig. S2, A–J), indicating that the Golgi disruption produced by α1 and α1 S47A are partially due to interactions with LIS1.

To determine if catalytic activity of α1 and α2 is also important for maintaining Golgi structure in vivo, independent of LIS1, cells were transfected with a double mutant that is both catalytically inactive and unable to bind LIS1 (α1 S47A/E38D; α2 S48A/E39D). These constructs also disrupted the Golgi and TGN (Fig. 2, H and I; Fig. S2, A–H).

The LIS1-binding mutant results suggest that catalytic activity is important, and that α subunits also compete for binding to LIS1 to regulate dynein-dependent Golgi structure, in agreement with previous reports (Ding et al., 2009). In addition, our results agree with Ding et al. (2009), who found that overexpression of α2 had a more dramatic effect on Golgi positioning. A role for LIS1 in Golgi structure has been suggested; LIS1+/− mouse embryonic fibroblasts had mild Golgi dispersal, and Cos-7 cells overexpressing LIS1 had more compact Golgi complexes (Smith et al., 2000).

Our results suggest a model whereby PAAFH Ib provides a link between the initiation of membrane tubule formation and the subsequent movement of these tubules along microtubules. PAAFH Ib α1 and α2, bound to LIS1, may initiate membrane curvature by intrinsic PLA2 activity, thus forming membrane tubules. Subsequently, LIS1 may switch to a LIS1–NdeI–dynein complex, facilitating the minus-end movement of membrane tubules along microtubules to the centrosome (Vallee and Tsai, 2006). This provides a mechanism to couple the formation of Golgi membrane tubules to dynein motors for the formation and maintenance of a centrally located, intact Golgi ribbon.

Loss of PAAFH Ib fragments the Golgi complex, inhibits tubule-mediated Golgi assembly, and reduces anterograde trafficking

To determine if PAAFH Ib α1 and α2 are required for Golgi structure and function, siRNA-mediated knockdown experiments were conducted. PAAFH Ib α1 and α2 can form catalytically active homo- and heterodimers (Manya et al., 1999). Therefore we used mixed siRNAs targeting both α1 and α2, reducing expression by 85.7 ± 3.3% and 81.8 ± 3.8% (n = 7 and 6; ± SEM), respectively, which did not affect levels of LIS1 (Fig. 3 A). As a consequence of α1 and α2 loss, the Golgi complex, ER-Golgi intermediate compartment (ERGIC), and the TGN became fragmented (Fig. 3, B–D; Fig. S3, A and B). Confocal microscopy revealed that fragmented Golgi puncta contained multiple cisternal markers, indicating that loss of α1 and α2 resulted in the formation of mini-stacks (Fig. 3 D; Fig. S3 C), which was confirmed by transmission EM (Fig. 3 E).

Similar fragmentation was seen with knockdown of LIS1, which could be rescued by expressing RNAi-resistant HA-LIS1 (Fig. 3, F–H), similar to other recent studies (Lam et al., 2010).

Membrane tubule–mediated assembly and maintenance of an intact Golgi ribbon is inhibited by PLA2 antagonists (de Figueiredo et al., 1999). To determine if knockdown of PAAFH Ib subunits similarly inhibits these tubules, we examined the reassembly of the Golgi during recovery from brefeldin A (BFA). After washout of BFA, the Golgi reassembles into separate mini-stacks that subsequently coalesce via membrane tubules into an intact ribbon (de Figueiredo et al., 1999). Knockdown of α1 and α2 or LIS1 had no apparent effect on BFA-stimulated retrograde movement of Golgi enzymes to the ER (unpublished data). Upon washout, the emergence of separate Golgi mini-stacks was unaffected by knockdown of PAAFH Ib subunits. In contrast, knockdown of α1 and α2 or LIS1 significantly inhibited subsequent coalescence into intact ribbons (Fig. 4, A–D),
Figure 2. PAFAH1b partially localizes to Golgi membranes and overexpression of catalytic subunits perturbs Golgi and TGN structure. (A) Confocal microscopy of cells mildly overexpressing α1 (HA tagged) and Rab6-GFP. Box indicates region in bottom panels. (B) Fluorescence intensity line plot of α1 and Rab6-GFP from bottom panel of A. (C) Western blot showing α1 and LIS1 cofractionate with Golgi (ManII) by sucrose-density gradient centrifugation. Bracket indicates nonspecific bands. (D) Confocal slice shows HA-LIS1 localizes around the Golgi (ManII). (E) α1 localizes to Golgi tubules. Arrowheads indicate colocalization with Rab6-GFP membrane tubules. (F) Line fluorescence intensity plot of LIS1 concentrated around Golgi complexes (from line in D). (G) Line fluorescence intensity of α1 on membrane tubules (from line in E). (H) HeLa cells transfected with the indicated α1 (HA tagged) and double labeled for HA and either a Golgi (GPP130) or TGN marker (M6PR). Overexpression of α1 wild type, α1 S47A (catalytic mutant), or α1 S47A/E38D (catalytic and LIS1-binding mutant) disrupted the structure of the Golgi and the TGN. Asterisks and arrowheads indicate transfected and untransfected cells, respectively. (I) Quantitation of HeLa and BRD cells with disrupted Golgi structure in cells transfected as indicated. Weighted means shown, n = 3; error bars = SEM; *, P < 0.05 compared with untransfected cells; **, P < 0.001 compared with α1 E38D (Behrens-Welch test). Bars: (A, bottom, and E) 5 μm; [A, top, D, and H] 10 μm.
PAFAH Ib subunits are important for maintaining an intact Golgi ribbon. (A) Western blot of PAFAH Ib subunits in control and α1/α2 siRNA-treated (72 h) BTRD cells. (B) Confocal stack of the Golgi (ManII) in control and α1/α2 siRNA-transfected cells. (C) The fraction of cells with fragmented Golgi. n = 7; error bars = SEM; P < 0.001 by t test. (D) 3D reconstructions of Golgi from confocal z-series of control and α1/α2 siRNA-treated cells visualized with anti-ManII (medial marker, red) and GalT-GFP (trans marker, green). (E) Electron micrographs from control and α1/α2 siRNA knockdown cells. (F) Western blot 48 h after transfection of control BTRD cells, LIS1 siRNA-transfected cells, and LIS1 knockdown cells overexpressing RNAi-resistant HA-LIS1. (G) Wide-field fluorescence image of the Golgi (ManII) in control and LIS1 siRNA-transfected BTRD cells. (H) Fraction of cells with fragmented Golgi. n = 3; error bars = SEM. Bars: (B) 2 µm; (D and E) 500 nm; (G) 10 µm.

which could be rescued by expressing RNAi-resistant LIS1 (Fig. 4, C and D). Importantly, unlike α1 and α2 knockdown, LIS1 knockdown Golgi mini-stacks displayed numerous membrane tubules, which were no longer seen when all three subunits were knocked down (Fig. 4, E and F). These results are consistent with our in vitro and overexpression studies and further support the conclusion that the catalytic α1 and α2 subunits of PAFAH Ib are capable of inducing membrane tubules in vivo. The membrane tubules that form in LIS1 knockdown cells are dependent on α1 and α2, but directed movement for mini-stack coalescence is inhibited in the absence of LIS1-mediated dynein interactions.

In addition to reassembly of the Golgi complex, membrane tubules are implicated in export from the TGN and secretion (Bard and Malhotra, 2006; De Matteis and Luini, 2008). Using ts045VSV-G as a transmembrane cargo, we found that transport from the ER to the Golgi complex was unaffected in α1 and α2 knockdown cells (Fig. 5, A and B). In contrast, export of ts045VSV-G from the TGN to the cell surface was significantly slowed in α1 and α2 knockdown cells (Fig. 5, A, C, and D). VSV-G transport was unchanged with LIS1 knockdown (Fig. 5 F), suggesting α1 and α2 have LIS1-independent roles in TGN-to-plasma membrane trafficking. The reduced transport of VSV-G with α1 and α2 knockdown could be partially rescued by the reintroduction of equivalent levels of RNAi-resistant α1, but not RNAi-resistant α1 S47A, indicating that PLA2 activity is important for regulating the transport of Golgi to plasma membrane cargo (Fig. 5 A, D; Fig. S2, I and J). Likewise, secretion of soluble cargo, ssHRP, was significantly inhibited in α1 and α2 knockdown cells (Fig. 5 E). Thus, loss of α1 and α2 may directly decrease TGN-to-plasma membrane tubulo-vesicular carriers (Bard and Malhotra, 2006; De Matteis and Luini, 2008).

Loss of α1 and α2 could also influence Golgi trafficking by affecting any number of components that require specific lipids for binding. Export from the TGN requires protein kinase D (PKD), which binds to membranes via diacylglycerol (Baron and Malhotra, 2002). A kinase-dead (KD) version of PKD, which inhibits TGN tubule fission (Bard and Malhotra, 2006), is a convenient marker for PKD association with the TGN. In α1 and α2 knockdown cells, PKD-KD-GFP underwent redistribution from the TGN to the cytoplasm and plasma membrane (Fig. S3 D). The structure and function of the Golgi and TGN is also dependent on COPI vesicles, AP-1 clathrin-coated vesicles, and microtubules;
however, we found that knockdown had no discernable effect on β-COP, AP-1, or tubulin (Fig. S3, E and F). Similarly, the COPII vesicle component Sec31 was unaffected (Fig. S3 E). These results suggest that α1 and α2 contribute to phospholipid remodeling that is important for PKD association with the TGN.

It is becoming clear that Golgi structure and membrane trafficking require constant and complex phospholipid remodeling. In addition to PAFAH Ib, cPLA2α has recently been shown to influence the Golgi by regulating tubule-mediated, cis-to-trans, inter-cisternal trafficking. Interestingly, loss of cPLA2α can be compensated by PAFAH Ib α1 (San Pietro et al., 2009). cPLA2α was also shown to regulate export from the TGN (Regan-Klapisz et al., 2009). Other recent studies found that another phospholipase, iPLA2γ, contributes to tubule-mediated retrograde trafficking from the Golgi (Morikawa et al., 2009). Although the molecular mechanisms are unclear, continual phospholipid remodeling by PLA2, PLA2 and lysophosphatidyl acyltransferase enzymes, such as LPAAT3/AGPAT3.
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- anti-\(\alpha\)1 and -\(\alpha\)2 in initial studies (K. Inoue, University of Tokyo, Tokyo, Japan); guinea pig and rabbit anti-\(\alpha\)1 and -\(\alpha\)2 antibodies (characterized here); rabbit anti-bovine CI-M6PR (by us [Brown and Farquhar, 1987]); rabbit anti-dynamin (MC63; M. McNiven, Mayo Clinic, Rochester, MN); rabbit anti–human GPP130 (A. Linstedt, Carnegie Mellon University, Pittsburgh, PA); rabbit anti-ManII (K. Moremen, University of Georgia, Athens, GA); chicken anti-\(\alpha\)2 (Abcam); mouse anti-LIS1 (Sigma-Aldrich); mouse anti-HA (Sigma-Aldrich and Covance); mouse anti–\(\beta\)-COP (BioMakor), mouse anti–\(\alpha\)-tubulin (Sigma-Aldrich), rabbit anti-Sec31A (W. Balch, Scripps Research Institute, La Jolla, CA); fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories, Inc. and Invitrogen); HRP-conjugated goat anti–chicken (Aves Laboratories), anti–guinea pig (Pocono Rabbit Farm and Laboratory), anti–rabbit (GE Healthcare), and anti-mouse (Invitrogen).

Preparation of plasmids

Bovine PAFAH Ib \(\alpha\)1 and \(\alpha\)2, pUC-P-\(\alpha\)1, and pUC-P-\(\alpha\)2 (Hattori et al., 1995) were gifts from Dr. Inoue. These were templates for \(\alpha\)1-S47A and \(\alpha\)2-S48A, which were made by PCR and ligation into pGEX6P1 (GE Healthcare) BamHI sites.

Tagging \(\alpha\)1 and \(\alpha\)2 on the N and C termini was problematic because such proteins were inactive. Although our polyclonal antibodies were useful for Western blots, we were unsuccessful in localizing endogenous \(\alpha\)1 and \(\alpha\)2 with many different polyclonal antibodies (made in rabbit, guinea pig, (Schmidt and Brown, 2009), may be critical for regulating the availability of curvature-altering lipids such as lysophospholipids, phosphatidic acid, diacylglycerol, and/or for the recruitment of other membrane-trafficking proteins, such as PKD (Bankaitis, 2009).

Our results demonstrate a novel role for PAFAH Ib PL\(\alpha\)2 \(\alpha\) subunits, which appear to be multi-functional, regulating PAF signaling, LIS1 function in dynein-mediated processes, and, as shown here, the formation of membrane tubules and the function of the Golgi complex. Our studies reveal a novel relationship of PAFAH Ib PL\(\alpha\)2 activity with dynein-dependent processes that are coupled by LIS1 to regulate Golgi structure. Additionally, our results show that \(\alpha\)1 and \(\alpha\)2 have a LIS1-independent role in export from the TGN.

Materials and methods

Materials

Sprague-Dawley male rats were from Charles River; BFA and cycloheximide from Enzo Life Sciences, Inc. Antibodies were as follows: rabbit anti-\(\alpha\)1 and -\(\alpha\)2 in initial studies (K. Inoue, University of Tokyo, Tokyo, Japan); guinea pig and rabbit anti-\(\alpha\)1 and -\(\alpha\)2 antibodies (characterized here); rabbit anti-bovine CI-M6PR (by us [Brown and Farquhar, 1987]); rabbit anti-dynamin (MC63; M. McNiven, Mayo Clinic, Rochester, MN); rabbit anti–human GPP130 (A. Linstedt, Carnegie Mellon University, Pittsburgh, PA); rabbit anti-ManII (K. Moremen, University of Georgia, Athens, GA); chicken anti-\(\alpha\)2 (Abcam); mouse anti-LIS1 (Sigma-Aldrich); mouse anti-HA (Sigma-Aldrich and Covance); mouse anti–\(\beta\)-COP (BioMakor), mouse anti–\(\alpha\)-tubulin (Sigma-Aldrich), rabbit anti–\(\gamma\)-adaptin (Santa Cruz Biotechnology, Inc.), rabbit anti–Sec31A (W. Balch, Scripps Research Institute, La Jolla, CA); fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories, Inc. and Invitrogen); HRP-conjugated goat anti–chicken (Aves Laboratories), anti–guinea pig (Pocono Rabbit Farm and Laboratory), anti–rabbit (GE Healthcare), and anti-mouse (Invitrogen).

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Fluorescence and electron microscopy

Cells were prepared as described previously (J. Figueiredo et al., 1999, 2001). Either GFP- or YFP-tagged proteins or secondary antibodies conjugated to Alexa 488, DyLight 488, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, or Cy5 were used. Coverslips were mounted with Vectashield (Vector Laboratories) mounting medium and imaged at room temperature. Wide-field epifluorescence was with a microscope (Axiohome II; Carl Zeiss, Inc.) with 40x or 100x Plan-Apochromat NA1.4 oil objectives, a digital camera (Orca II; Hamamatsu Photonics), and Openlab software (PerkinElmer). Spinning disk confocal images were from a microscope (Eclipse TE2000U; Nikon) with Plan-Apo 60×/NA1.4 or Plan-Apo100×/NA1.4 oil objectives, with an Ultrascan LC (PerkinElmer), a camera (1394 ORCA-ER; Hamamatsu Photonics), and Ultraview software (PerkinElmer). A spinning disk microscope (DMI6000B; Leica) equipped with an oil 63×/NA1.4 objective, 3i Marianas spinning disk system, Photometrics HQII CCD camera, and 3i Slidebook 5.0 software was also used (Intelligent Imaging Innovations). For electron microscopy, FEI Morgagni 268 transmission EM was used.

VSV-G-YFP and ssHRP-Fleg
tos45SVSVG-YFP pixel intensities of the juxtaplasmic Golgi region and total cell were measured from confocal images using ImageJ (National Institutes of Health, Bethesda, MD), with appropriate background fluorescence subtraction (per area). Kinetic equations for tos45SVSVG-YFP transport were fit by least squares nonlinear regression using Origin 3.5 software, based on first order kinetic equations (Yamaguchi et al., 1998). For ssHRP-flag measurements, media and lysate HRP activity was quantified by absorbance, using 3, 3‘, 5, 5‘-tetramethylbenzidine reagent (Sigma-Aldrich), and normalized to total HRP expression.

Image analysis and statistics

The Golgi disruption indices were by categorizing Golgi as diffuse, fragmented, or intact. For most experiments, ≥ 300 cells were counted for each condition, and each was repeated independently at least three times. The statistical significance between control and knockdown cells was with a two-tailed, unequal variance t-test. The significance of overexpression phenotypes was by Behrens-Welch testing. Images and z-projections were cropped, brightness adjusted, or contrast adjusted using ImageJ, Photoshop CS3, or 3i Slidebook 5.0 software. 3D reconstruction was done using Velocity software (PerkinElmer). Line intensity plots were done using 3i Slidebook 5.0 software (Intelligent Imaging Innovations).

Online supplemental material

Fig. S1 shows the localization of α1, α2, α2 S48A, or α1 S47A/E383D to multiple Golgi cisternae and TGN membrane tubes. Fig. S2 shows Golgi and TGN structure is disrupted upon overexpression of α1, α2, and various mutant α1 and α2 versions in HeLa and BTRD cell lines. Fig. S3 shows fragmentation of the ERGIC and TGN, and that the TGN is associated with Golgi mini-stacks with loss of α1 and α2 in siRNA knockdown experiments. Fig. S3 shows vesicle markers and microtubules are unaffected, whereas PKD-KD-GFP localization is changed upon α1 and α2 knockdown. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908105/DC1.

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