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PAFAH Ib Phospholipase A₂ Subunits Have Distinct Roles in Maintaining Golgi Structure and Function

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Abstract

Recent studies showed that the phospholipase subunits of Platelet Activating Factor Acetylhydrolase (PAFAH) Ib, $\alpha 1$ and $\alpha 2$, partially localize to the Golgi complex and regulate its structure and function. Using siRNA knockdown of individual subunits, we find that $\alpha 1$ and $\alpha 2$ perform overlapping and unique roles in regulating Golgi morphology, assembly, and secretory cargo trafficking. Knockdown of either $\alpha 1$ or $\alpha 2$ reduced secretion of soluble proteins, but neither single knockdown reduced secretion to the same degree as knockdown of both. Knockdown of $\alpha 1$ or $\alpha 2$ inhibited reassembly of an intact Golgi complex to the same extent as knockdown of both. Transport of VSV-G was slowed but at different steps in the secretory pathway: reduction of $\alpha 1$ slowed *trans* Golgi network to plasma membrane transport, whereas $\alpha 2$ loss reduced endoplasmic reticulum to Golgi trafficking. Similarly, knockdown of either subunit alone disrupted the Golgi complex but with markedly different morphologies. Finally, knockdown of $\alpha 1$, or double knockdown of $\alpha 1$ and $\alpha 2$, resulted in a significant redistribution of kinase dead protein kinase D from the Golgi to the plasma membrane, whereas loss of $\alpha 2$ alone had no such effect. These studies reveal an unexpected complexity in the regulation of Golgi structure and function by PAFAH Ib.

Keywords

PLA₂; PAFAH Ib; Golgi complex; secretory trafficking

1.0 INTRODUCTION

A series of pharmacological studies originally showed that cytoplasmic phospholipase A (PLA) activities regulate the structure and function of the Golgi complex [1-4]. More recent studies have revealed an unexpectedly large number of cytoplasmic PLA enzymes involved in these processes, with a common theme of partial localization to the Golgi complex and functional regulation of the Golgi complex by controlling the shape and formation of cisternal membranes as well as transport carriers exiting the Golgi complex [5, 6]. The

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calcium-dependent enzyme cPLA₂ α was shown to translocate to the Golgi during times of increased secretory load, induce membrane tubules that bridge between Golgi cisternae for intra-cisternal trafficking of secretory cargo, and regulate export from the *trans* Golgi network (TGN) [7-9]. An unrelated cytoplasmic phospholipase, iPLA₁ γ , is located on *cis* Golgi and ER-Golgi-Intermediate Compartment (ERGIC) membranes and may facilitate anterograde traffic through the Golgi stack [10, 11]. PLA2G6 mediates membrane tubule formation and organization of the ERGIC [12]. Finally, recent studies have determined that a fourth, unrelated phospholipase, Platelet Activating Factor Acetylhydrolase Ib (PAFAH Ib), also regulates the functional organization of the Golgi complex [13]. PAFAH Ib regulates membrane tubule formation that leads to the coalescence of the Golgi complex into an intact ribbon structure and regulates export from the TGN. Thus, at least four different PLA enzymes function at different domains of the Golgi to regulate its structure and function.

Besides contributing to Golgi structure and function, PAFAH Ib appears to have multiple biological roles. PAFAH Ib consists of homo- or heterodimers of two PLA₂ subunits α 1 and α 2, and a dimer of a third subunit, LIS1 [14-16]. PAFAH Ib was first discovered by its ability to hydrolyze the extracellular signaling lipid, PAF, which plays a role in platelet activation and inflammation [17]. LIS1, the causative agent of the fatal brain disorder Miller-Dieker lissencephaly, functions independently of the α subunits to regulate the activity of cytoplasmic dynein [18-20]. Although the functional relationship between PAFAH Ib and LIS1/dynein is unclear, several studies strongly suggest that altering the cytoplasmic levels of α 1 and α 2 controls the amount of LIS1 that is available for functional interactions with dynein [13, 21, 22].

Another area of uncertainty is the function of the different α 1 and α 2 subunit combinations. Human α 1 and α 2 are 63% identical at the amino acid level and form catalytically active homo- and heterodimers [23]. However, numerous studies have established that α 1 and α 2 have differences in substrate preferences [23], catalytic rates when bound to LIS1 [23], developmental expression patterns [24], and neurological phenotypes in different mutant backgrounds [25].

Previous studies have suggested a role for PLA₂ activity in regulating the formation of membrane tubules that facilitate cargo export from the Golgi complex and endosomes [1]. Using a cell-free reconstitution system and biochemical fractionation of bovine brain cytosol [26], we discovered that PAFAH Ib was able to induce tubule formation from Golgi membranes, and in vivo studies showed that α 1 and α 2 are partially localized to Golgi membranes [13]. Unable to find a cell line that expresses only one of the α subunits, we performed double knockdown experiments of PAFAH Ib α 1 and α 2 subunits, which resulted in the fragmentation of the Golgi/TGN ribbon into smaller, disconnected cisternal stacks (mini-stacks) and significantly decreased export from the TGN. Recent studies have also found that PAFAH Ib regulates the formation of membrane tubules and receptor recycling from endosomes [27]. However, these studies left the open question of the relative roles of α 1 and α 2 in regulating the functional organization of the Golgi complex. Here we report that single knockdown of either α 1 or α 2 results in significant differences in Golgi morphology and function. We conclude that α 1 and α 2 have overlapping and distinct

functions in regulating the Golgi, indicating an additional level of complexity for the role of these enzymes at the Golgi complex.

2.0 MATERIALS AND METHODS

BFA and cycloheximide were from Biomol Research Laboratories, Inc. Antibodies and GFP-tagged constructs were obtained as follows: guinea pig anti- α 1 antibody (by us ([13]); chicken anti- α 2 (Abcam, Inc.); mouse anti-HA (Covance); rabbit anti-dynamin (MC63) (M. McNiven, Mayo Clinic, Rochester, MN); rabbit anti-ManII (K. Moremen, Univ. of Georgia, Athens, GA); mouse anti- β -COP (BioMakor), mouse anti- α tubulin (Sigma Chemical Co.), rabbit anti- γ -adaptin (Santa Cruz), rabbit anti-Sec31A (W. Balch, Scripps Research Institute, La Jolla, CA) fluorescent secondary antibodies (Jackson Immuno-Research Laboratories and Invitrogen); HRP-conjugated goat anti-chicken (Aves Laboratories), anti-guinea pig (Pocono Rabbit Farm and Laboratory), anti-rabbit (GE Healthcare), and anti-mouse (Gibco). ERGIC53-GFP was from C. Fromme (Cornell University, Ithaca, NY); ts045 VSVG-YFP was from B. Storrie (University of Arkansas for Medical Sciences, Little Rock, AR); ssHRP-Flag (in pEN1) and PKD-KD-GFP (in pEGFP) were from V. Malhotra (Centre de Regulació Genòmica, Barcelona, Spain). RNAi-resistant PAFAH Ib α 1 was generated as previously described [13].

2.1 Cell Culture, Transfection and RNAi

BTRD bovine testicular were cultured in MEM (Mediatech, Inc.) with 10% BGS in a 37°C, 95% humidity, and 5% CO₂ incubator. Cells were obtained from the Diagnostic Laboratory at the Cornell College of Veterinary Medicine and have been used extensively for studies on secretory and endocytic trafficking [13, 27]. Double stranded RNA purchased from Thermo Scientific were: AGAAUGGAGAGCUGGAACAUAU and GGAGAACUGGAGAAUAUAUAU, for α 1 and α 2, respectively [13]. The control double stranded RNA was siGenome non-targeting siRNA #1 and #2 from Thermo Scientific. 30 nM of RNA was transfected on two consecutive days with Lipofectamine RNAiMax (Invitrogen). DNA transfection was carried out 48 h after the first RNA transfection, using Lipofectamine 2000 (Invitrogen) with ¼ the amount of reagent and DNA indicated by the manufacturer. Experiments were conducted 72 h after the initial RNA transfection.

2.2 Cell Lysates and Immunoblotting

Cells were lysed with 0.05% TritonX-100 in phosphate buffered saline with a protease inhibitor cocktail (Roche) and scraped from the culture dish. Nuclei and membranes were pelleted with low speed centrifugation. Soluble proteins were separated by SDS-PAGE, followed by subsequent Western blotting with indicated antibodies, and detection with chemiluminescent reagent (Millipore).

2.3 Fluorescence and Electron Microscopy

Cells were prepared as described [3, 28]. Either GFP or YFP tagged proteins or the indicated primary antibodies in combination with secondary antibodies conjugated to Alexa 488, DyLight 488, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate were used. Coverslips were mounted with Vectashield (Vector Laboratories) mounting media and

imaged at room temperature. Wide-field epifluorescence images were taken with a Zeiss Axioscope II, a Zeiss 40x Plan-Apochromat NA1.4 oil objective lens, a Hamamatsu Orca II digital camera, and Openlab software (Improvision). Spinning disk confocal images were from a Nikon Eclipse TE2000-U, Nikon Plan-Apo 60x/NA1.4 or Nikon Plan-Apo100x/NA1.4 oil objectives, with a Perkin-Elmer Ultraview LCI, a Hamamatsu 1394 ORCA-ER camera, and Perkin-Elmer Ultraview software. For electron microscopy, a FEI Morgagni 268 transmission EM was used.

2.4 VSV-G-YFP and ssHRP-Flag

The pixel intensities of ts045 VSV-G-YFP for entire cells and the juxtanuclear Golgi region were measured from confocal images using ImageJ (NIH), with background fluorescence subtraction (per area). For ssHRP-Flag measurements, HRP activity was quantified by absorbance using 3, 3', 5, 5'-tetramethylbenzidine reagent (Sigma). Collected media and cell lysates were used for measurements, which were normalized to total HRP expression (total activity in the media + lysate).

2.5 Image Analysis and Statistics

For experiments, 100 cells were counted for each condition, and each experiment was repeated independently three times. The Golgi morphologies were categorized and counted blind to determine the fraction of 'intact' ribbon Golgi, highly fenestrated, 'webbed' Golgi, or fragmented Golgi. Fragmented Golgi were further categorized to include enlarged, spherical fragments, categorized as dilated, and punctate, small fragments classified as vesiculated. The statistical significance between control and knockdown cells was determined with a two-tailed, unequal variance T-test. Images and z-projections were cropped, brightness adjusted, or contrast adjusted using ImageJ or Photoshop CS3.

3.0 RESULTS

3.1 Knockdown of $\alpha 1$ or $\alpha 2$ Subunits Fragments the Golgi Complex with Different Morphologies

To examine the contribution of individual PAFAH Ib catalytic subunits to regulation of Golgi structure, we performed single knockdown studies of $\alpha 1$ or $\alpha 2$. Cultured BTRD cells express PAFAH Ib $\alpha 1$ and $\alpha 2$ subunits at approximately equal levels, composing 0.025-0.05% of the total soluble protein weight (Fig. S1). Knockdown of either $\alpha 1$ or $\alpha 2$ alone resulted in a significant loss of the appropriate subunit but not the other (Fig. 1A). Similar to double knockdown, loss of either $\alpha 1$ or $\alpha 2$ resulted in fragmentation of the Golgi ribbon but with qualitatively different morphologies. As visualized by immunofluorescence with the medial marker α -mannosidase II (ManII), the Golgi in $\alpha 1$ knockdown cells were often dilated, ranging from distinct round fragments to web-like or highly fenestrated membranes, although still clustered in the juxtanuclear region (Figs 1B, S2). These results were quantified revealing a significant proportion of fragmented and dilated Golgi ribbons in $\alpha 1$ knockdown cells (Fig. 1C, D). Thin section transmission electron microscopy (TEM) revealed that the fragments seen by fluorescence microscopy were relatively normal, stacked (mini-stack) structures but with obviously dilated cisternae (Figs 1F, S2).

Knockdown of $\alpha 2$ also produced fragmented Golgi ribbons, but in contrast to $\alpha 1$, these fragments appeared as short linear and punctate vesiculated membranes by immunofluorescence (Figs 1B-D, S2). By TEM, the Golgi observed were mini-stacks with significantly vesiculated cisternae (Figs 1F, S2). Strikingly, $\alpha 2$ knockdown cells also exhibited fairly dilated ER morphology as seen by EM (Fig. S2).

Knockdown of $\alpha 1$ or $\alpha 2$ also resulted in fragmentation of the ERGIC or altered distribution of ERGIC53 as seen by ERGIC53-GFP, with $\alpha 2$ knockdown cells showing the most dramatic effect in comparison to $\alpha 1$ knockdown cells or knockdown of both $\alpha 1$ and $\alpha 2$ (Fig. 1E). Additionally, $\alpha 2$ knockdown cells appeared to have a higher proportion of ERGIC53-GFP fluorescence in ER-like structures compared to control and other knockdown cells (Fig. 1E).

3.2 Reduced $\alpha 1$ or $\alpha 2$ Levels Slow Secretory Trafficking But at Different Steps of the Pathway

Loss of both $\alpha 1$ and $\alpha 2$ inhibits transport through the secretory pathway, at least at the level of export from the TGN [13]. To determine the relative contribution of each subunit, knockdowns of individual subunits were performed, revealing an unexpected difference between the two with regard to secretory trafficking. When measuring bulk soluble protein transport through the secretory pathway, by assaying soluble secreted HRP (ssHRP) from transfected cells, knockdown of either $\alpha 1$ or $\alpha 2$ resulted in a loss of secretion that was approximately 50% that of double knockdown (Fig. 2A). To more accurately assess the location of secretory protein trafficking inhibition by $\alpha 1$ or $\alpha 2$ knockdown, we employed the ts045 VSV-G-YFP protein. ts045 VSV-G-YFP was allowed to accumulate in the ER at the restrictive temperature (40°C), shifted to 20°C to allow export from the ER and accumulation in the TGN, and then shifted again to the permissive temperature (32°C) to allow export from the TGN and delivery to the cell surface. In control cells, ts045 VSV-G clearly accumulated in the intact ribbon-like TGN by 120 min following shift to 20°C and was then delivered to the plasma membrane following shift to 32°C for 80 min (Fig. 2B). In $\alpha 1$ knockdown cells, ts045 VSV-G similarly accumulated in the fragmented TGN and was significantly slowed in export and delivery to the plasma membrane following shift to 32°C (Fig. 2B, C). This inhibition was rescued by expression of siRNA-resistant $\alpha 1$ construct after knockdown (Fig. 2C). In $\alpha 1$ knockdown cells, but not in controls, VSV-G was observed to remain in the Golgi, even after 80 min following shift to 32°, as determined by colocalization with ManII (Fig. S3A-F). Quantitation of this kinetic inhibition of VSV-G delivery to the cell surface is shown in Fig. S3G.

In contrast to $\alpha 1$, knockdown of $\alpha 2$ resulted in a significant inhibition of ts045 VSV-G reaching the TGN following shift to 20°C, and a prolonged presence in the ER (Fig. 2B). After 120 min at 20°C ~40% of $\alpha 2$ siRNA-treated cells had a substantial amount of VSV-G in the ER. However, once in the TGN, transport to the plasma membrane was not noticeably slowed (compare control versus $\alpha 2$ siRNA at the 80 min time point) (Fig. 2B, C). Even at 80 min following shift to 32°C, ~15-20% of cells still had VSV-G in the ER. Given these results, it was not surprising that knockdown of both $\alpha 1$ and $\alpha 2$ produced an inhibition

equal to that of $\alpha 1$ knockdown alone (Fig. 2C). Thus, it would appear that $\alpha 1$ and $\alpha 2$ affect different trafficking steps to regulate transport dynamics.

3.3 Knockdown of $\alpha 1$ or $\alpha 2$ Does Not Affect Coat Protein Localization But Has Differential Effects on the Localization of Protein Kinase D

We previously found that knockdown of both $\alpha 1$ and $\alpha 2$ did not significantly impact the localization of various trafficking proteins such as the COPI subunit β -COP, clathrin AP-1 adaptor, or the COPII subunit Sec31 [13]. Likewise, single knockdown did not influence these markers (Fig. 3A). Protein kinase D (PKD) is a regulator of TGN secretory vesicle fission [29]. A kinase dead version of PKD (PKD-KD) localizes to the TGN and induces a dominant negative effect on vesicle fission, thereby causing the formation of extensive membrane tubules [30]. We found that double knockdown appeared to inhibit PKD-KD-induced TGN tubule formation, and resulted in a significant relocation of PKD-KD from the TGN to the plasma membrane [13] (Fig. 3B). Interestingly, cells subjected to single knockdown of $\alpha 1$, but not $\alpha 2$, exhibited a similar phenotype (Fig. 3B). Thus, $\alpha 1$ subunits alone play a role in maintaining PKD-KD at the TGN.

3.4 Both $\alpha 1$ and $\alpha 2$ Are Required for Assembly of an Intact Golgi Ribbon

In addition to secretory trafficking, PLA₂ activity controls the biogenesis and maintenance of an intact Golgi ribbon [1]. Biogenesis of the Golgi complex has often been studied using recovery from brefeldin A (BFA) treatment as a model system [3], and we have previously shown that knockdown of both $\alpha 1$ and $\alpha 2$ inhibits the coalescence of mini-stacks into an intact ribbon [13]. Similarly, following knockdown of either $\alpha 1$ or $\alpha 2$ and recovery from BFA treatment, the Golgi complex reassembled into separate mini-stacks, but their coalescence into intact ribbons was inhibited (Fig. 4A, B). In each case, the Golgi reassembled into mini-stacks during recovery from BFA, but the Golgi did not form intact ribbon morphology (Fig. 4C), demonstrating that reassembly of the Golgi requires both subunits.

4.0 DISCUSSION

In previous studies, we found that double knockdown of the two catalytic subunits of PAFAH1b, $\alpha 1$ and $\alpha 2$, produced a severely fragmented Golgi complex and inhibited secretion [13]. Here we examine $\alpha 1$ and $\alpha 2$ subunits individually and show that they play overlapping but also distinct roles in regulating Golgi structure and function. This is a surprising result because $\alpha 1$ and $\alpha 2$ can form both functional homo- and heterodimers, and all subunit combinations can form competent complexes with LIS1 [23].

Loss of either α subunit produces distinctly different Golgi morphologies: the Golgi complexes in $\alpha 1$ knockdown cells are swollen and highly fenestrated, whereas $\alpha 2$ knockdown cells exhibit a severe vesiculated phenotype. Interestingly, the Golgi morphologies in single knockdown cells are not exactly the same as those in double knockdown cells, in which the fragmented Golgi complexes do not show the same degree of cisternal dilation or vesiculation [13]. These results strongly suggest that Golgi morphology requires a tight balance between the levels of both $\alpha 1$ and $\alpha 2$ subunits. The reason for the

differences in single knockdown cells is not clear. One possibility could be that $\alpha 2$ appears to have a higher affinity for LIS1 (based on the relative abilities of $\alpha 1$ and $\alpha 2$ to compete with Ndel for binding to LIS1) [21]. This difference in affinity is likely relevant for regulation of LIS1 and dynein function in vivo; however, cells with reduced $\alpha 2$ have smaller, vesiculated Golgi and more dilated ER, which is not consistent with $\alpha 2$ knockdown only affecting dynein activation. Dynein activity is important for anterograde ER to Golgi trafficking [31], which should increase in the absence of $\alpha 2$ competition for binding LIS1. On the contrary, $\alpha 2$ knockdown phenotypes suggest decreased ER to Golgi transport. A second possibility to explain differing phenotypes between $\alpha 1$ and $\alpha 2$ knockdown is by their relative substrate preferences. In vitro studies suggest that heterodimers and $\alpha 1/\alpha 1$ homodimers are more selective for phosphatidic acid (1-*O*-alkyl-2-acetyl-3-*sn*-glycerophosphoric acid) species and exhibit little to no activity on phosphatidylethanolamine, whereas $\alpha 2/\alpha 2$ homodimers have much lower phosphatidic acid catalytic activity and had preference for PAF, a phosphatidylcholine derivative (1-alkyl-2-acetoyl-*sn*-glycero-3-phosphocholine), and phosphatidylethanolamine species (1-*O*-alkyl-2-acetyl-3-*sn*-glycero-phosphorylethanolamine) [23]. The generation of distinct lysophospholipid species may help to account for changes in membrane architecture by changing membrane shape and/or feeding into different lipid metabolic pathways [32, 33]. However, it is still unclear what the relative contributions of $\alpha 1/\alpha 2$ phospholipase activities and influences on LIS1 might be to regulating the functional organization of the Golgi complex, which is an interesting issue that has so far been difficult to tease apart.

Loss of either $\alpha 1$ or $\alpha 2$ inhibited soluble protein secretion to ~50% that achieved by double knockdown, initially suggesting that each has overlapping functions in secretion. However, the second surprising finding was that $\alpha 1$ or $\alpha 2$ appear to facilitate protein trafficking at different stages of the secretory pathway—loss of $\alpha 2$ resulted in slowed export from the ER and delivery to the TGN, whereas loss of $\alpha 1$ inhibited binding of PKD-KD to the TGN and export from the TGN. These observations are consistent with previous pharmacological studies showing that PLA₂ antagonists have similar effects on ER and TGN export [34]. This result is also consistent with the dilated ER and Golgi morphologies for $\alpha 2$ and $\alpha 1$ knockdown, respectively. These results suggest that $\alpha 2$ homodimers could function early in the secretory pathway and $\alpha 1$ homodimers later, or that loss of $\alpha 2$ has a greater affect than $\alpha 1$ on the retrograde recycling of key proteins required for anterograde trafficking. Moreover, it is possible that these different effects on trafficking could be indirectly related to the differences in Golgi architecture produced by single knockdowns. Our localization studies of expressed subunits are not helpful in this regard because we observed no significant difference in the intracellular distributions of $\alpha 1$ and $\alpha 2$, with both being cytosolic, nuclear, and Golgi-associated [13].

PAFAH Ib $\alpha 1$ and $\alpha 2$ subunits both have profound effects on secretory trafficking, Golgi structure, and the assembly of the Golgi complex. BFA washout studies show that reduced levels of either subunit delays the reassembly of an intact Golgi ribbon to the same extent as knockdown of both subunits. Interestingly, $\alpha 2$ knockdown, while having an effect on cargo export from the ER does not show an observable difference in the appearance of Golgi mini-stacks from the ER. Instead, either $\alpha 1$ knockdown or $\alpha 2$ knockdown inhibits reassembly at

the stage of mini-stack coalescence into Golgi ribbon morphology, a stage that has been observed to require dynein-mediated membrane tubule transport [3, 35].

Importantly cells did not compensate for single knockdowns by increasing expression of the other subunit. Even though single knockdowns still expressed wild type levels of the other subunit, it was not enough to overcome the loss of one subunit. Moreover, knockdown of $\alpha 1$ and $\alpha 2$ had no effect on Lis1 levels [13]. Thus, the overall functional organization of the Golgi is very sensitive to total $\alpha 1$ and $\alpha 2$ levels.

Work from several laboratories in the past year has revealed an unexpected level of complexity with regard to phospholipase regulation of the Golgi complex. In addition to PAFAH Ib, two other phospholipases have recently been shown to regulate different aspects of Golgi structure and function. These include cPLA₂ α which regulates the balance between the formation of Golgi membrane tubules and COPI coated vesicles [8, 9] as well as export from the TGN [7], and PLA2G6, which regulates ERGIC structure [12]. Our studies here strongly suggest that PAFAH Ib $\alpha 1$ and $\alpha 2$ homodimers have some distinct functions, indicating that the Golgi is influenced by four phospholipases. The extent to which these phospholipases function independently to control Golgi structure and function is unclear. Although, previous studies indicate that loss of cPLA₂ α can be compensated by PAFAH Ib $\alpha 1$: VSV-G trafficking in cells from cPLA₂ $\alpha^{-/-}$ mice is only decreased with the additional reduction of $\alpha 1$ levels by RNAi [8]. It is evident that future studies will be required to dissect the individual and combined roles of these enzymes.

The molecular mechanisms by which PAFAH Ib, iPLA₁ γ , or cPLA₂ α regulate the functional organization of the Golgi complex is unclear. The combined evidence strongly suggests that all three can influence the shape of Golgi membranes by controlling membrane tubule formation. Moreover, it would appear that continual phospholipid remodeling by PLA₁, PLA₂, and lysophospholipid acyltransferase enzymes, such as LPAAT3/AGPAT3 [36], 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 and vesicle-associated coat proteins [32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- PLA₂ regulation of the Golgi complex is more complicated than previously expected
- PAFAH Ib $\alpha 1$ and $\alpha 2$ have overlapping and unique roles in regulating the Golgi complex
- Loss of $\alpha 1$ or $\alpha 2$ have different effects on Golgi morphology and function

Figure 1.

Single knockdown of either PAFAH Ib $\alpha 1$ or $\alpha 2$ causes differential fragmentation of the Golgi complex. (A) Western blots showing amounts of $\alpha 1$ and $\alpha 2$ in control, single, or double siRNA-treated BTRD cells 72 h after initial siRNA transfection.

Dynamin served as a loading control. (B) Confocal z-series of the Golgi (anti-ManII immunofluorescence). (C) The morphological changes in Golgi structure were quantified ‘blind’, counted as normal Golgi (intact), webbed Golgi ribbons ($\alpha 1$ siRNA panel on the left) or fragmented ($\alpha 1$ siRNA panel on the right). (D) Quantification of fragmented Golgi, subcategorized into fragmented (not swollen or shrunken), dilated (swollen), and vesiculated (shrunken). (E) Direct wide-field fluorescence of ERGIC53-GFP in control, single, and double knockdowns. Scale bars = 5 μ m. (F) Transmission electron microscopy of thin sections from control and single knockdown BTRD cells. Scale bars = 500 nm.

Figure 2.

Secretory protein trafficking is inhibited at different steps in single PAFAH Ib $\alpha 1$ or $\alpha 2$ knockdown cells. (A) Kinetics of ssHRP secretion, measured by HRP activity in the media of control or siRNA-treated (as indicated) BTRD cells. Values were normalized to expression levels of HRP and HRP activity in control cells at 8 h. (B) Confocal images of ts045 VSV-G-YFP transport in single knockdown cells. Transfected cells were incubated at 40°C to accumulate ts045 VSV-G-YFP in the ER, shifted to 20°C for 20 or 120 min to allow export from the ER and transport to the TGN, and then shifted to 32°C for 80 min to allow export from the TGN and transport to the cell surface. (C) Quantification of the percent of total ts045 VSV-G-YFP fluorescence on the PM following shift to 32°C for 80 min. For $\alpha 2$ knockdown cells, only cells with VSV-G outside the ER (~80% of cells) were measured.

Figure 3.

Localization of vesicle markers and protein kinase D in single knockdown cells. (A) Knockdown of PAFAH Ib $\alpha 1$ or $\alpha 2$ does not detectably affect clathrin AP-1 adaptor, COPI subunit β -COP, or the COPII subunit Sec31a in BTRD cells. Scale bars = 5 μ m for AP1 and β -COP, 10 μ m for Sec31a. (B) Knockdown of $\alpha 1$, but not $\alpha 2$, causes a loss of PKD-KD-GFP from the TGN and redistribution to the plasma membrane. Scale bars = 10 μ m.

Figure 4.

Golgi reassembly following BFA washout is inhibited in PAFAH Ib $\alpha 1$ or $\alpha 2$ single knockdown cells. (A) Confocal images of control and cells treated with siRNA against individual or both subunits. BTRD cells were treated with media containing 5 $\mu\text{g/mL}$ BFA for 20 min, followed by replacement with BFA-free media to allow reassembly of the Golgi. Cells were fixed at the indicated time points (after BFA removal) and processed for immunofluorescence with anti-ManII. Scale bars = 10 μm . (B) Quantification of reassembly into an intact Golgi ribbon in control and siRNA-treated cells. Error bars = SEM. (C) Confocal micrographs illustrating the morphology of the Golgi complex (anti-ManII immunofluorescence) during recovery from BFA in control and siRNA treated cells. Scale bars = 2 μm .