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PAFAH Ib phospholipase A_2 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, α1 and α2 partially localize to the Golgi complex and regulate its structure and function. Using siRNA knockdown of individual subunits, we find that α1 and α2 perform overlapping and unique roles in regulating Golgi morphology, assembly, and secretory cargo trafficking. Knockdown of either α1 or α2 reduced secretion of soluble proteins, but neither single knockdown reduced secretion to the same degree as knockdown of both. Knockdown of α1 or α2 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 α1 slowed *trans* Golgi network to plasma membrane transport, whereas α2 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 α1, or double knockdown of α1 and α2, resulted in a significant redistribution of kinase dead protein kinase D from the Golgi to the plasma membrane, whereas loss of α2 alone had no such effect. These studies reveal an unexpected complexity in the regulation of Golgi structure and function by PAFAH Ib. This article is part of a Special Issue entitled Phospholipids and Phospholipid Metabolism.

1. 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 calcium-dependent enzyme $cPLA_2\alpha$ was shown to translocate to the Golgi during times of increased secretory load, induce membrane tubules that bridge between Golgi cisternae for intracisternal trafficking of secretory cargo, and regulate export from the *trans* Golgi network (TGN) [7–9]. An unrelated cytoplasmic phospholipase, $iPLA_1\gamma$, 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 PLA_2 subunits α1 and α2, and a dimer of a third subunit, LIS1 [14–16]. PAFAH Ib was first discovered for 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 α1 and α2 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 α1 and α2 subunit combinations. Human α1 and α2 are 63% identical at the amino acid level and form catalytically active homo- and heterodimers [23]. However, numerous studies have established that α1 and α2 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 PLA_2 activity in regulating the formation of membrane tubules that facilitate cargo export

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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 α1 and α2 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 α1 and α2 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 α1 and α2 in regulating the functional organization of the Golgi complex. Here we report that single knockdown of either α1 or α2 results in significant differences in Golgi morphology and function. We conclude that α1 and α2 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. Materials and methods

BFA and cycloheximide were from Biomol Research Laboratories, Inc. Antibodies and GFP-tagged constructs were obtained as follows: guinea pig anti-α1 antibody (by us [13]); chicken anti-α2 (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 α1 was generated as previously described [13].

2.1. Cell culture, transfection and RNAi

BTRD bovine testiclular cells were cultured in MEM (Mediatech, Inc.) with 10% BGS in a 37°C, 95% humidity, and 5% CO_2 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: AGAAUGGAGAGCUGGAACAUU and GGAGAACUGGAGAAUAUUAUU, for α1 and α2, 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, or 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 40× 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 60×A/N1.4 or Nikon Plan-Apo100×/N1.4 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. Results

3.1. Knockdown of α1 or α2 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 α1 or α2. Cultured BTRD cells express PAFAH Ib α1 and α2 subunits at approximately equal levels, composing 0.025–0.05% of the total soluble protein weight (Fig. S1). Knockdown of either α1 or α2 alone resulted in a significant loss of the appropriate subunit but not the other (Fig. 1A). Similar to double knockdown, loss of either α1 or α2 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 α1 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 α1 knockdown cells (Fig. 1C, D). Thin section transmission electron microscopy (TEM)