PKCη is required for β1γ2/β3γ2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus

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Protein kinase D (PKD) binds to a pool of diacylglycerol (DAG) in the TGN and undergoes a process of activation that involves heterotrimeric GTP-binding protein subunits βγ to regulate membrane fission. This fission reaction is used to generate transport carriers at the TGN that are en route to the cell surface. We now report that PKD is activated specifically by G protein subunit β1γ2 and β3γ2 via the Golgi apparatus–associated PKCη. Compromising the kinase activity of PKCη-inhibited protein transport from TGN to the cell surface. Expression of constitutively activated PKCη caused Golgi fragmentation, which was inhibited by a kinase inactive form of PKD. Our findings reveal that βγ, PKCη, and PKD act in series to generate transport carriers from the TGN and their overactivation results in complete vesiculation of the Golgi apparatus.

Introduction

We identified a marine natural product ilimaquinone (IQ), which converted the Golgi apparatus into small vesicles (Takizawa et al., 1993). We reasoned that IQ treatment overactivates the membrane fission reaction, thus converting the Golgi apparatus into small vesicles. If true, an understanding of the IQ-mediated Golgi vesiculation process should reveal components of the membrane fission reaction. IQ-mediated Golgi vesiculation was reconstituted in permeabilized cells and was shown to require trimeric G proteins and a serine/threonine kinase called protein kinase D (PKD) (Jamora et al., 1997, 1999). Of interest is the revelation that these components are also required for the formation of transport carriers at the TGN that are destined for the plasma membrane (Maeda et al., 2001; Baron and Malhotra, 2002; Liljedahl et al., 2001; Yeaman et al., 2004). Our findings so far can be summarized as follows. During protein transport, a pool of diacylglycerol (DAG) is generated, which recruits PKD to a specific site on the TGN. Gβγ activates PKD. Activated PKD recruits and/or activates downstream targets, which leads to membrane fission.

To understand the mechanism of PKD-mediated transport carrier formation and regulation of this process, we have sought to identify the specific βγ subunits involved in this process. Of the βγ combinations tested, only β1γ2 and β3γ2 were found to activate PKD. Interestingly, this activation is through a member of the PKC family of kinases called PKCη. We describe here the regulation of transport carrier formation via βγ, PKCη, and PKD and the significance of this regulation for the overall organization of the Golgi apparatus.

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Abbreviations used in this paper: DAG, diacylglycerol; IQ, ilimaquinone; PKD, protein kinase D; VSV, vesicular stomatitis virus.

Results

Identification of βγ subunits that regulate Golgi organization

βγ combinations were selected using one of five β subunits fused with FLAG tag (β1-β5), and one of five γ subunits fused with HA tag (γ1-γ5). 25 combinations of these two proteins were transfected, separately, into HeLa cells (Fig. 1 A). The organization of the Golgi apparatus was monitored by immunofluorescence microscopy, using antibodies against GM130 and TGN46 as markers for the cis-medial Golgi and the TGN, respectively. Of all the βγ combinations tested, β1γ2 and β3γ2 caused Golgi breakdown in greater than 60% of transfected cells (Fig. 1 A; and Fig. 1 B, a and b).

To confirm the role of β1γ2 and β3γ2 subunits in Golgi apparatus organization, a mutant of γ2 subunit was generated by replacing cysteine 68 with serine in the carboxy-terminal portion of the protein (γ2C68S). This change alters the CAAX box and prevents geranylgeranylation of γ2-C68S subunit, inhibiting membrane association. Transfected β1γ2-C68S and β3γ2-C68S subunits were cytosolic, and the Golgi complex appeared intact in all transfected cells (Fig. 2, a and b).

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A

	γ1	γ2	γ3	γ4	γ5
β1	+	+++	-	-	-
β2	-	-	-	-	-
β3	+	+++	-	-	-
β4	-	-	-	-	-
β5	-	+	-	-	-

B

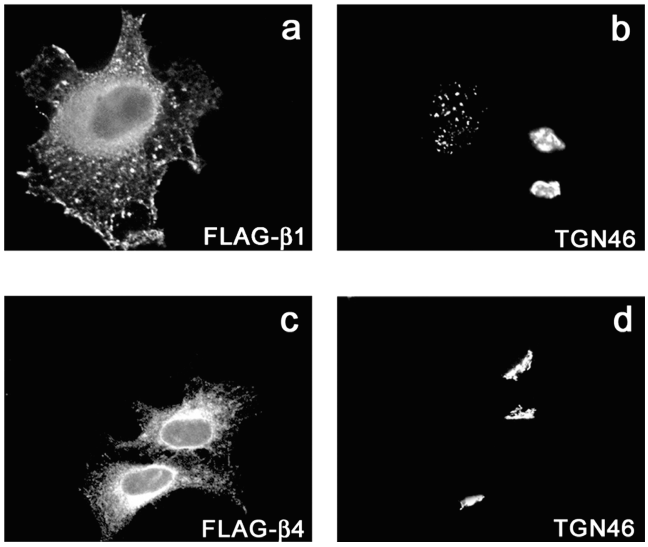

Figure 1. **β1γ2 and β3γ2 expression fragments the Golgi membranes in intact cells.** (A) Various combinations of tagged βγ (FLAG-β and HA-γ) were coexpressed in HeLa cells and the organization of the Golgi apparatus monitored by fluorescence microscopy using antibodies to the early (GM130) and late (TGN46) Golgi cisternae. 200 cells were counted in four different experiments. The percentage of cells with fragmented Golgi apparatus is >60% (+++), with β1γ2 and β3γ2 between 20 and 60% (+) and other βγ combinations <20% (−). (B) HeLa cells were transfected with FLAG-β1 and HA-γ2 (a and b), or FLAG-β4 and HA-γ2 (c and d). The cells were visualized by fluorescence microscopy with anti-FLAG (a and c) and anti-TGN46 (b and d) antibodies, respectively. Expression of β1γ2 and β3γ2 fragments the Golgi apparatus.

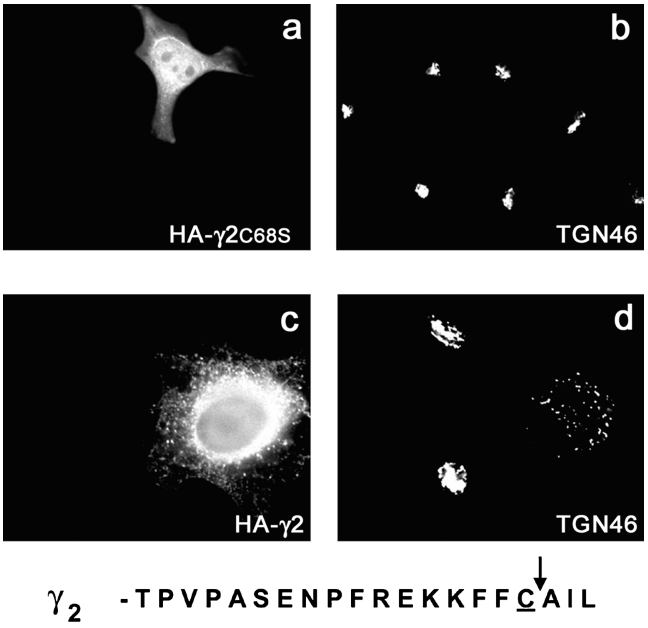

Figure 2. **Effect of γ2 prenylation on β1γ2 mediated Golgi fragmentation in intact cells.** HeLa cells were transfected with FLAG-β1 and HA-γ2C68S (a and b) or HA-γ2 (c and d). The sequence of the carboxy-terminal 20 amino acids of γ2 in which a specific cysteine is replaced with serine to generate γ2-C68S is shown. The site at which geranylgeranyl transferase-I adds the geranylgeranyl residue to the γ2 subunit is highlighted with an arrow. The cells expressing the wild-type and the mutant form of γ2 were probed by fluorescence microscopy with anti-HA (a and c) and anti-TGN46 (b and d) antibodies, respectively.

Recombinant histidine-tagged β1γ2 and β3γ2 were expressed in human 293T cells, purified, and added to permeabilized NRK cells. β1γ2 and β3γ2 (10 nM) specifically caused fragmentation of the Golgi apparatus in >60% of cells (Fig. 3, a and b). In contrast, 10 nM β1γ2-C68S and β3γ2C68S or β4γ2 had no effect on the organization of the Golgi apparatus (Fig. 3, c–f).

We have shown before that the PH domain of PKD blocks Golgi fragmentation induced by total bovine brain Gβγ (Jamora et al., 1999). We tested the effect of PKD-PH domain on Golgi fragmentation induced by β1γ2 and β3γ2, respectively. The GST-tagged PH domain of PKD and GFP-tagged PH domains of PLCβ2 and PLCδ1, were cotransfected along with β1γ2 or β3γ2 in HeLa cells. The PH domains of PKD and PLCβ2 are predominantly cytosolic, whereas the PH domain of PLCδ1 is localized to the plasma membrane, via PI $(4,5)P_2$ binding (data not shown). The organization of the Golgi apparatus was monitored by fluorescence microscopy. The PH domains of PKD and PLCβ2 inhibited β1γ2- and β3γ2-mediated Golgi breakdown (Fig. 4 A). The latter result is consistent with the findings that PLCβ2 PH domain binds βγ (Wang et al., 2000). No inhibition of Golgi fragmentation was observed by PLCδ1 PH domain, which binds βγ on plasma membrane only in the presence of PI(4,5)P2 (Wang et al., 1999). PH domains of Akt/PKB and Bruton's tyrosine kinase did not have effect on Golgi fragmentation induced by βγ (Fig. 4 A).

β1γ2 and β3γ2 activate protein kinase D

Since βγ-mediated effects on the Golgi apparatus are reportedly mediated through activation of PKD, we tested the effect of βγ on PKD phosphorylation and activation in intact cells. HeLa cells were transfected with GST-tagged wild-type PKD and FLAG-β1HA-γ2 or FLAG-β4HA-γ2 as a negative control. GST-PKD was immunoprecipitated from cells and tested for its activation. As shown in Fig. 4 B, PKD autophosphorylation at Ser916 was detected with all the constructs tested, but the phosphorylation of the activation loop at Ser744-748 was increased in the presence of β1γ2 (Fig. 4 B, lane 1). Phosphorylation of the activation loop was inhibited by the PH domains of PLCβ2 and PKD, but not by the PH domain of PLCδ1 (Fig. 4 B, lanes 3, 4, and 5). β3γ2 had similar effects on the activation of PKD (data not shown). β4γ2 did not activate phosphorylation of PKD in the activation loop (Fig. 4 B, lane 2). Based on these results, we conclude that β1γ2 induces phosphorylation and activation of PKD in intact cells.

β1γ2-mediated activation of PKD is via PKCη

It is known that phosphorylation in the activation loop of PKD is mediated by PKC (Waldron et al., 2001). PKCη and PKCε

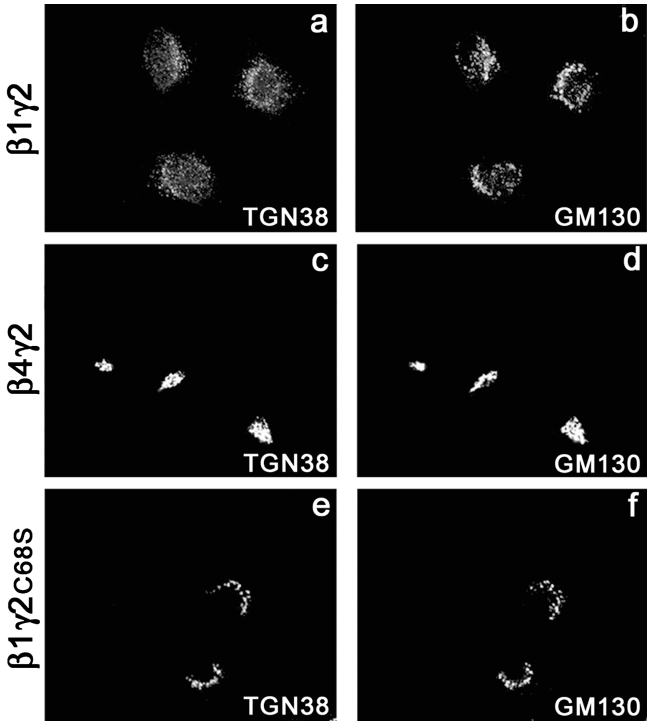

Figure 3. **Purified β1γ2 subunits cause Golgi fragmentation in semi-permeabilized NRK cells.** Purified recombinant His-tagged β1γ2 (a and b), β4γ2 (c and d), and β1γ2C68S (e and f) were added to permeabilized NRK cells. 30-min after incubation, the cells were fixed and visualized for immunofluorescence with anti-TGN38 (TGN marker) (a, c, and e), and anti-GM130 (cis-medial Golgi marker) (b, d, and f) antibodies.

both activate PKD. These PKC isoforms are highly homologous and localize to the Golgi apparatus (Goodnight et al., 1995; Lehel et al., 1995; Brandlin et al., 2002; Rey et al., 2004). PKCη binds to the PH domain of PKD (Waldron et al., 1999), and we have shown previously that addition of the PH domain of PKD inhibits IQ- and βγ-mediated Golgi fragmentation (Jamora et al., 1999). To test a role for PKCη in secretion mediated by the βγ-PKD pathway, various combinations of FLAG-β1HA-γ2, GST-PKD wt, FLAG-PKCη wt, and GFP-PKCε wt were coexpressed in HeLa cells. GST-PKD wt was immunoisolated and probed for phosphorylation at Ser744/748 (activation loop) and Ser916 (autophosphorylation site) by Western blotting. Using phosphospecific antibodies, coexpression of β1γ2 and PKCη resulted in a fourfold increase in phosphorylation of Ser744/748 of PKD compared with other combinations tested (Fig. 5 C, lane 2). Interestingly, although both PKCη and PKCε are Golgi localized, β1γ2 activated PKCη but not PKCε (Fig. 5, A and B, first two lanes).

PKCη regulates TGN export

Our findings so far illustrate the significance of PKCη in the βγ-mediated activation of PKD. But is PKCη required for protein transport from the TGN? To test this, HeLa cells were transfected with a kinase-inactive form of PKCη and temperature-sensitive mutant form of the vesicular stomatitis virus (VSV)–G protein with a GFP tag. At the nonpermissive temperature of 39.5°C, the VSV–G protein is synthesized and re-

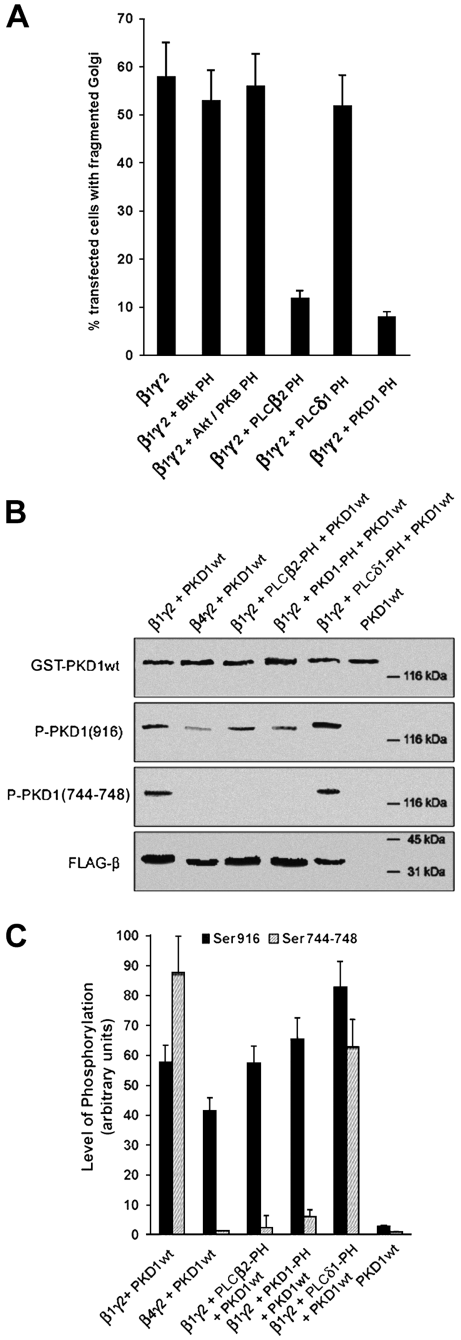

Figure 4. **Effect of PH domains on the β1γ2-dependent Golgi fragmentation and PKD phosphorylation.** (A) β1γ2-mediated Golgi fragmentation is inhibited by specific PH domains in intact cells. HeLa cells were transfected with FLAG-β1HA-γ2 and GFP-PH domains of proteins listed. The organization of the Golgi apparatus was monitored by fluorescence microscopy using anti-TGN46 and -GM130 (late and early Golgi specific markers) antibodies. 200 cells expressing FLAG-β1 were counted to determine the percentage of cells with fragmented Golgi membranes. Percentage of cells transfected and levels of protein expression for each PH domain was similar in all experiments as monitored by immunofluorescence and Western blotting with anti-GFP antibody, respectively. (B and C) Effect of β1γ2 on PKD phosphorylation in the activation loop. HeLa cells were cotransfected with the constructs listed. GST-PKD was immunoprecipitated and analyzed by Western blotting (B) with antibodies against GST,phospho-PKD (Ser916), phospho-PKD (Ser744–748), respectively, and quantitated by densitometric scan (C). Anti-FLAG antibodies were used to monitor the expression level of β1γ2 and β4γ2 in the respective cell extracts (B). Values are means (±SD, vertical bars) of three separate experiments.

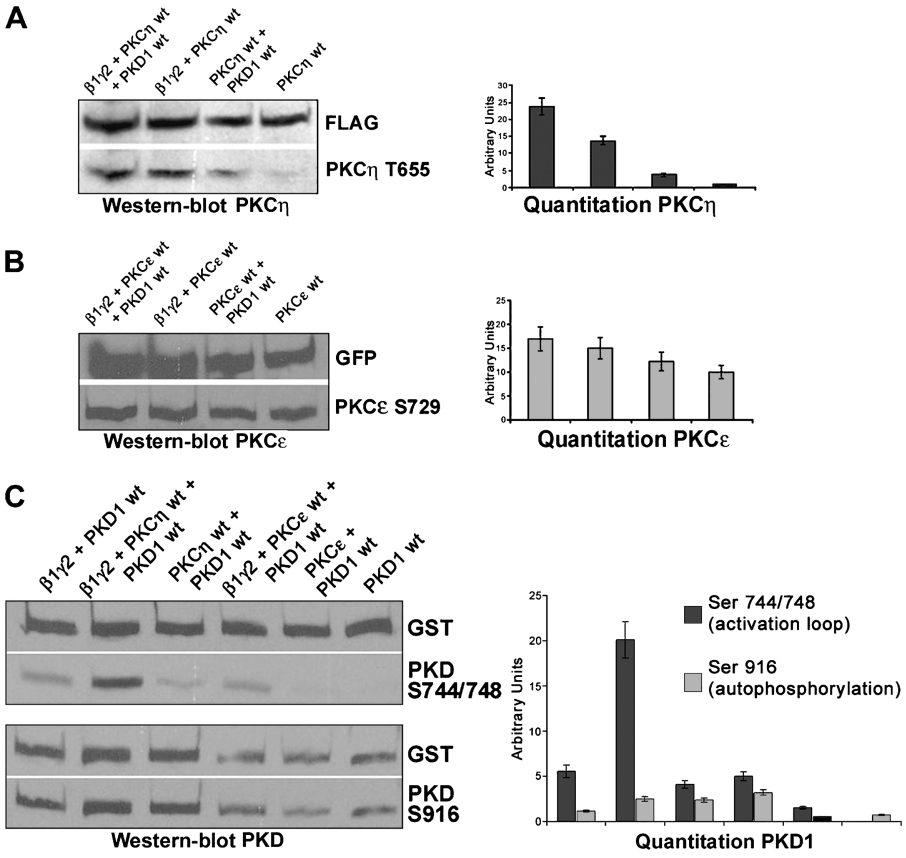

Figure 5. **Activation of PKCη by β1γ2, and subsequent hyper phosphorylation of PKD in the activation loop.** HeLa cells were cotransfected with the constructs listed. The cells were lysed and the extracts analyzed by Western blotting to monitor the phosphorylation status of FLAG-PKCη, GFP-PKCε, and GST-PKD, respectively. The blots were quantitated as described in Materials and methods. (A) For PKCη, the antibody used recognizes threonine 655 (T655). (B) Similar experimental procedure was used to monitor the effect of β1γ2 expression on the phosphorylation status of PKCε phosphorylation (Ser729). (C) Coexpression of β1γ2 and PKCη caused a fourfold increase in the phosphorylation of Ser744/748 (in the activation loop) of PKD without any appreciable change in the autophosphorylation of Ser916 (lane 2, shown in the Western blot and the bar graph). Values are means (±SD, vertical bars) of three separate experiments.

tained in the ER because of folding defects. Upon shift to 20°C, the protein leaves the ER and is retarded in the TGN. Subsequent shift to 32°C releases VSV-G from the TGN and it is transported to the cell surface. As shown in Fig. 6, in control cells expressing wild-type PKCη, VSV–G protein is found at the cell surface 120 min after releasing the G protein from the TGN by a shift to 32°C (Fig. 6, a–d). In cells expressing PKCη kinase inactive, VSV–G protein was still in the Golgi, even after 2 h at 32°C (Fig. 6, e–h).

Wild-type and constitutively activated forms of PKCη were expressed in HeLa cells and the organization of the Golgi apparatus was monitored by fluorescence microscopy using antibodies to the early (GM130) and the late Golgi (TGN46). The wild-type PKCη localized to the Golgi, and at the light microscopy level, the Golgi membranes appeared largely unaltered in organization (Fig. 7, a and b). Golgi membranes in cells expressing the constitutively activated form of PKCη are, however, highly fragmented and appear diffusely dispersed in the

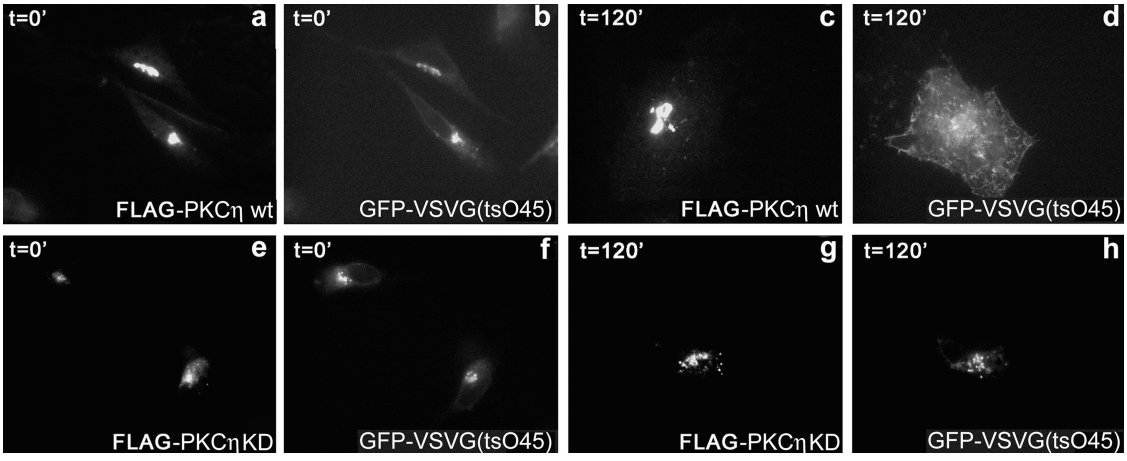

Figure 6. **PKCη is required for protein transport from TGN to the cell surface.** HeLa cells were transfected with GFP-tsO45 VSV-G and FLAG-PKCη-wt or FLAG-PKCη-kinase dead. Following the procedure described in Materials and methods, immunofluorescence microscopy was used to detect GFP-tsO45 VSV-G localization. VSV–G protein is retained in the Golgi apparatus in PKCη kinase dead–expressing cells after 120 min incubation at the permissive temperature C (e–h), compared with cells expressing the wild-type PKCη (a–d).

cytoplasm (Fig. 7, c and d). The organization of other cellular compartments was unaffected under these conditions (data not shown). Coexpression of PKD-KD inhibited PKCη-CA–mediated Golgi fragmentation and these kinases were found to colocalize to the Golgi and the tubules emanating from the TGN (Fig. 7, e–h, and insets).

There are at least 10 isoforms of typical PKC family of kinases. Mice lacking a member of this class of kinases are viable. This is most likely due to redundancies amongst these kinases. Interestingly, mice lacking PKCη are defective in wound healing and epithelial cell architecture (Chida et al., 2003). Membrane transport is necessary for these processes and this phenotype fits well with the requirement of PKCη and its downstream target, PKD, in Golgi to cell surface transport. PKCη and PKCε are highly homologous and can activate PKD. It is possible that in the absence of PKCη, PKCε carries out this function. Additionally, because of their slow turnover, we have not been able to achieve significant depletion of the endogenous levels of PKCη by siRNA.

PKD, AKAP-Lbc, and the Golgi connection

As shown previously and described here, PKD is a cytosolic protein, which binds Golgi membranes through interaction with DAG (Baron and Malhotra, 2002). The Golgi-associated pool of PKD is in an activated form and required for membrane fission (Maeda et al., 2001). PKD also has a plasma membrane associated target called Kidins220 in neurons (Iglesias et al., 2000). More recently, Scott and colleagues have reported a potentially interesting connection between PKD and a protein known as AKAP-Lbc (Carnegie et al., 2004). AKAP-Lbc is proposed to be a scaffold for three different kinases in this scheme. It has been reported that AKAP-Lbc binds PKA, PKCη, and PKD. When cells expressing these kinases exogenously are treated with PdBu, PKCη is activated, which in turn activates PKD as monitored by an antibody against the phosphorylated Ser744/748-PKD. Activation of PKA by forskolin/IBMX was shown to phosphorylate AKAP-Lbc, which then releases activated PKD into the cytoplasm. But do these interactions have a physiological significance? Cells expressing GFP-AKAP-Lbc were cotransfected with GST-PKD wild type or GST-PKD kinase dead. The cells were then treated with PdBu, or PdBu and forskolin/IBMX, by the procedure of Scott and colleagues (Carnegie et al., 2004). The localization of PKD and AKAP-Lbc was monitored by fluorescence microscopy using antibodies to the respective tags. In untreated cells, wild-type PKD was clearly visible on Golgi membranes. AKAP-Lbc, however, does not colocalize with the TGN-specific PKD (Fig. 8 d). Treatment of cells with PdBu caused translocation of PKD and AKAP-Lbc to the cell surface. Imaging by confocal microscopy revealed a partial colocalization of these proteins on the plasma membrane (Fig. 8 h and inset). The Golgi membranes under these conditions, however, were intact. In other words, PdBu dependent activation of PKD was not sufficient for vesiculation of the Golgi apparatus. Treatment of cells with PdBu and forskolin/IBMX did not change the overall localization of these two components. They remained on the plasma membrane although the two proteins appeared by confocal microscopy to be separated from each other (Fig. 8 l). The same results were found with the kinase-dead form of PKD and AKAP-Lbc (Fig. 8, a–c, e–g, and i–k). Our results show that PKD and AKAP-Lbc do not colocalize on Golgi membranes and treatment with PdBu alone or PdBu and forskolin is not sufficient to activate PKD in a form capable of Golgi vesiculation.

Discussion

We have revealed a requirement for a new kinase, PKCη, and specific βγ subunits, β1γ2, and β3γ2 in the PKD-dependent fission of Golgi membranes. Gβγ and PKD have been shown previously to regulate the production of transport vesicles from the TGN (Jamora et al., 1997, 1999; Liljedahl et al., 2001; Yeaman et al., 2004). PKCη is shown here to be required for pro-

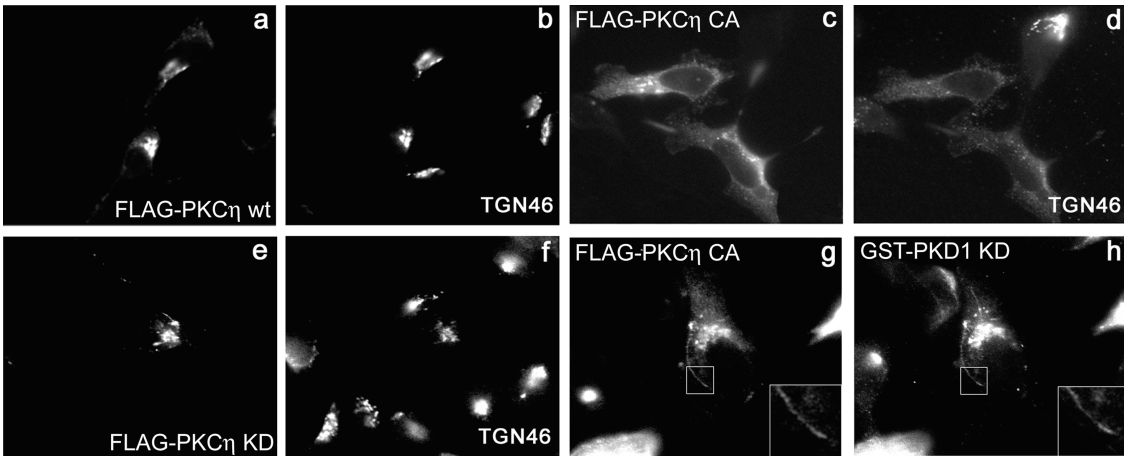

Figure 7. **Expression of constitutively activated PKCη fragments the Golgi apparatus.** HeLa cells were transfected with tagged versions of FLAG-PKCη wt (a and b), FLAG-PKCη constitutive active (c and d) and FLAG-PKCη constitutive active + GST-PKD kinase dead (e–h). The localization of the respective proteins was monitored by fluorescence microscopy using specific anti-tag antibodies. PKCη-wt is localized to the Golgi apparatus (a and b). The Golgi apparatus in cells expressing PKCη constitutive active is fragmented (c and d). The PKCη constitutive active–mediated Golgi fragmentation is inhibited upon expression of PKD kinase dead (e and f). Interestingly, PKD kinase dead and PKCη constitutive active colocalize both at the level of Golgi cisternae (g–h) and the emanating tubules (insets in g and h). CA denotes constitutively activated, and KD a kinase-dead form of the respective kinase.

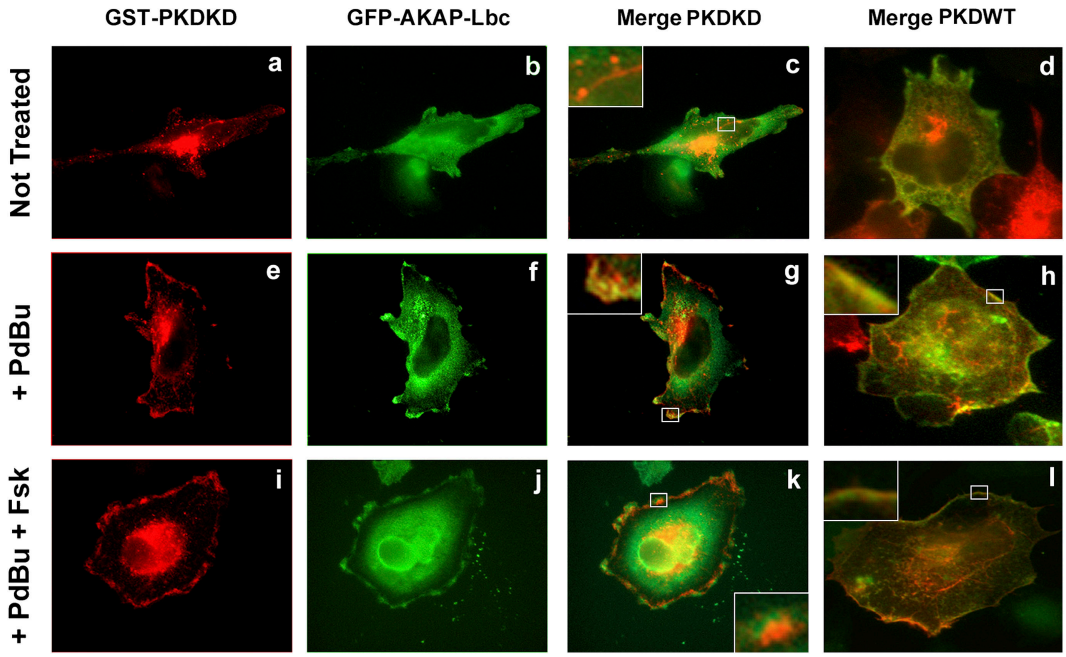

Figure 8. **PKD on the Golgi membranes does not colocalize with AKAP-Lbc.** HeLa cells were transfected with GFP-AKAP-Lbc and either wild-type (d, h, and l) or kinase-dead form of GST-PKD (a–c, e–g, and i–k). The location of AKAP-Lbc and PKD was monitored by confocal microscopy using GFP and anti-GST antibodies, respectively. PKD wild type and kinase dead is localized to the TGN (d) and the TGN-derived tubes, in the case of kinase-dead PKD (c and inset). The Golgi-associated form of PKD is in an activated state but it does not colocalize with AKAP-Lbc. Treatment with PdBu activates both the wild-type and the kinase-dead form of PKD. However, the PdBu-activated form of PKD is not sufficient to induce Golgi fragmentation. The PdBu-activated PKD translocates to the plasma membrane and partially colocalizes with AKAP-Lbc (g and h). Forskolin/IBMX treatment retains activated PKD on the plasma membrane, although it is not in complex with AKAP-Lbc (k and l).

tein transport from the TGN. There are three different isoforms of PKD in mammalian cells and all are specifically involved in the transport of cargo carrying a baso-lateral sorting signal (Yeaman et al., 2004). Knockdown of PKD by siRNA has been reported to block secretion of neurotensin (Li et al., 2004). We have shown previously that inhibiting PKD kinase activity blocks secretion by preventing membrane fission (Liljedahl et al., 2001). Long tubules containing cargo destined to the cell surface are seen emanating from the TGN under these conditions. Conversely, as shown here, overactivation of PKD by βγ or PKCη (by expressing β1γ2 or a constitutively activated form of PKCη) causes en mass fragmentation of the Golgi apparatus. This fragmentation is inhibited by expressing a kinase-inactive PKD. Together, our findings reveal that β1γ2, PKCη, and PKD act in series to promote membrane fission to generate transport carriers from the TGN. Overactivation of this reaction vesiculates the entire Golgi apparatus. These findings underscore the significance of the regulated production of transport carriers. We believe this equilibrium has to exist, otherwise the Golgi apparatus risks being converted into small vesicles by membrane fission during normal protein transport.

It has recently been shown that AKAP-Lbc acts as a scaffold, which recruits PKCη to activate both PKA and PKD (Carnegie et al., 2004). The activated PKD is dissociated from the complex. The site at which this reaction is performed within the cell, however, is not clear. We have shown before that PKA is not involved in PKD-dependent membrane fission and transport carrier formation (Jamora et al., 1999). Although this is a fascinating scheme for spatial activation of kinases, the physiological connection between AKAP-Lbc, PKCη, and PKD vis a vis membrane fission is not obvious from these findings. We show here that PKD does not colocalize with AKAP-Lbc on Golgi membranes. It is possible that PKD-AKAP-Lbc interact in a biologically relevant process at the cell surface. However, PKD-dependent Golgi vesiculation is independent of its potential interaction with AKAP-Lbc.

A number of components have been reported to be required for transport from the Golgi apparatus to the cell surface. The notable candidates: Par1 (Cohen et al., 2004), Lim kinase (Rosso et al., 2004), dynamin (Yang et al., 2001), PI4K (Audhya et al., 2000; Bruns et al., 2002; Levine and Munro 2002), cdc42 (Musch et al., 2001), myosin (Musch et al., 1997), kinesin (Kreitzer et al., 2000), and BARS-50 (Weigert et al., 1999). Cdc42, Par1, Lim kinase, kinesin, and dynamin are all reportedly involved in transport to the apical surface. These proteins are not likely to participate in βγ-PKCη-PKD-DAG–dependent membrane fission pathway, which is specialized for transport to the basolateral surface or for proteins carrying basolateral sorting signals (Yeaman et al., 2004). BARS-50 or CtBP (transcriptional corepressor) is reported to posses a membrane fission activity (Weigert et al., 1999). This is an exciting addition to the list of components involved in trafficking cargo. However, the details by which BARS-50—or, in its absence, another such component—might function in this reaction is not clear (Hildebrand and Soriano, 2002; Hidalgo Carcedo et al., 2004). Yeast PIK1 and mammalian

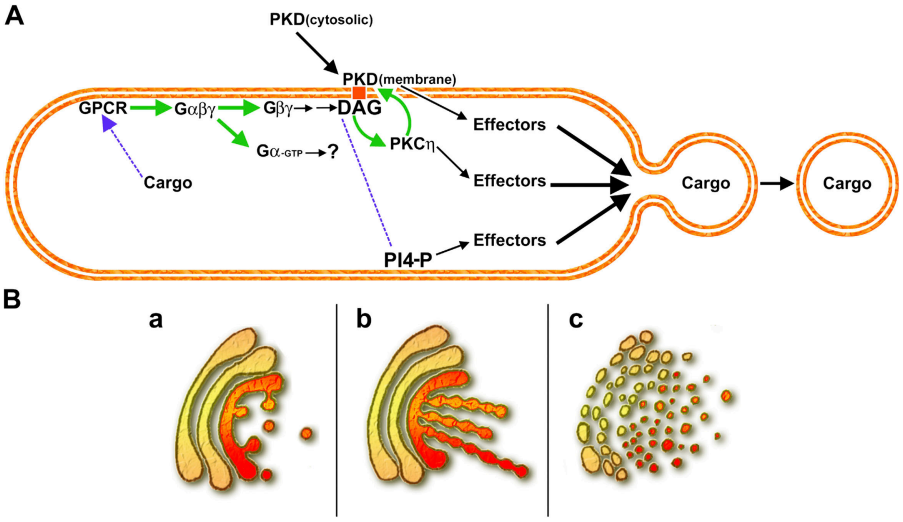

Figure 9. **Generation of transport carriers from the TGN: a working hypothesis**. (A) We propose that through a cargo-dependent involvement of G protein coupled receptor (GPCR), a trimeric G protein is activated at the TGN. The Gβγ subunits are involved in the production of DAG. The exact role of Gα in this process remains unclear. DAG activates PKCη and recruits PKD to the TGN. PKCη activates the TGN-bound PKD. The identity of Golgi-associated GPCR and the mechanism by which βγ results in the production of DAG are currently not known. Targets of PKCη, in addition to PKD, and the downstream targets of PKD are not known. It is possible that these kinases activate a diacylglycerol kinase that converts DAG into phosphatidic acid (PA) and a phospholipase, which convert PA into lysophosphatidic acid (LPA). LPA in our model would destabilize the neck of the budding transport carrier and promote fission. Green arrows denote activation; thin black arrows indicate involvement of additional components; and blue dotted line is suggestive of a hypothetical connection. (B) The scheme outlined above is used to regulate the formation of transport carriers from the TGN (a). Inactivation of PKCη or PKD does not affect cargo sorting and packaging, however membrane fission is inhibited, thus generating cargo filled transport carriers as large tubules attached to the TGN (b). Overactivation of PKCη and its downstream target PKD over activates the fission reaction, and the entire TGN is converted into small vesicles; The vesiculated TGN is an inactive acceptor for transport carriers from the preceding Golgi cisternae, and this combination generates a vesiculated Golgi apparatus (c).

PI4K are involved in events leading to the formation of transport carriers from the Golgi apparatus (Audhya et al., 2000; Bruns et al., 2002; Godi et al., 2004). Although PKD has been shown to coprecipitate with a PI4K activity, the identity of that lipid kinase is not known (Nishikawa et al., 1998). Sec14p in *S. cerevisiae* regulates DAG levels, DAG reportedly activates an ARF-GAP Gcs1p, and these components are required for Golgi-to–cell surface transport in yeast (Bankaitis and Morris, 2003). It is clear that phosphoinositides and DAG are both required for protein transport from theTGN to the cell surface. Rather than playing a direct role in the final fission events, we propose that phosphoinositides and DAG act to nucleate the fission events. These effectors participate in processes such as cargo packaging, changing the membrane curvature, constricting the emerging bud and cutting the membrane at the appropriate time, when cargo of the correct size and quantity has been encapsulated into the newly forming carriers. These effectors would also be involved in recruiting the correct molecular motors to direct transport carriers for their onward journey to the plasma membrane. But the identity of the effectors and how they are made to work in concert to produce a transport carrier of the right size, shape and the number is yet to be determined (see Fig. 9 for a working hypothesis).

Inactivation of PKD only inhibits transport from the TGN to the basolateral surface (Yeaman et al., 2004). Why does its overactivation (or treatment of cells with IQ, which led to the identification of PKD) cause complete fragmentation of the Golgi apparatus? A likely possibility is that when PKD is overactivated, components of the fission machinery continuously form transport carriers from the TGN until it is reduced to small vesicles. The vesiculated TGN, we propose, is inactive as an acceptor for transport carriers arriving from the preceding Golgi cisternae. The continued generation of transport carriers from the preceding cisternae, combined with a vesiculated TGN, generates a fragmented Golgi apparatus under these conditions.

The Golgi complex in mammalian cells has a "complex organization" that is maintained amidst a tremendous flux of membranes during protein transport. The mechanism of protein transport across, and out of the Golgi apparatus, is likely to be regulated by a variety of signals and layers of checks and balances. We have described four components (β1γ2, β3γ2-PKCη, PKD, and DAG) that control membrane fission. Many other factors will also be required to generate transport carriers from the TGN in a controlled manner to accommodate cellular demands (Fig. 9).

Materials and methods

cDNA constructs

pCDNA3.1 plasmids expressing FLAG-β1 to β5, and HA-γ1 to γ5, and with histidine tags were a gift from S. Gutkind (National Institutes of Health [NIH], Bethesda, MD). pEGFP constructs expressing PLCδ1, Akt/PKB, and Btk PH domains were donated by D. Alessi (University of Dundee, Scotland, UK). GFP-AKAP-Lbc plasmid was a gift from John D. Scott (Vollum Institute, Portland, OR). PKD plasmids were constructed in our laboratory by Yusuke Maeda (University of Osaka, Japan). HA-γ2C68S mutant was generated by PCR, replacing the 68th codon, TGC (cysteine) with AGC (serine). PLCβ2 PH domain and full-length PKCε wild type were amplified by RT-PCR using purified HeLa cells RNA, and cloned in the vector pEGFP-C1 (CLONTECH Laboratories, Inc.). FLAG-PKCη was a gift from Motoi Ohba (Showa University, Tokyo, Japan). Mutagenesis of both PKCs was done following the manufacturer's protocol for the QuikChange site-directed mutagenesis kit (Stratagene). The final products were PKCη A160E (PKCη constitutive active), PKCη K384A (PKCη kinase dead), PKCε T566E (PKCε constitutive active), and PKCε K437W (PKCε kinase dead).

Cell culture and transfections

HeLa ATCC and 293T cells were cultured in DMEM (Cellgro), supplemented with 10% fetal bovine serum (GIBCO BRL). NRK cells were grown in α modification of Eagle's medium (α-MEM; Cellgro).

HeLa ATCC cells were transfected by established procedures by either calcium phosphate (Sambrook et al., 1989) or lipofectamine 2000 (as recommended by Invitrogen). For both procedures, a final concentration of 4 μg or 0.5 μg, respectively, of plasmid or combination of plasmids were transfected into 1.8×10^5 cells/well in 500 μl of media grown on coverslips previously coated with pronectin F (Biosource International). Cells were fixed after 24 h of transfection for immunofluorescence microscopy.

Protein purification
293T cells were transfected with βγ constructs with histidine tags by the calcium phosphate method into 20 150-mm plates. Cells were washed 48 h after transfection with PBS and incubated for 30 min on ice with 1 ml of lysis solution (50 mM NaH_2PO_4, 500mM NaCl, 1% CHAPS, 10 mM imidazole).

The cells were passed through a series of decreasing pore size gauge needles (18G11/2, 20G11/2, and 25G5/8) 20 times each, and lysed in a Dounce homogenizer on ice. Cell lysates were cleared by centrifugation at 10,000 rpm for 30 min in a refrigerated centrifuge. The expressed proteins were purified using nickel-agarose columns and eluted from the column with imidazole following the manufacturer's protocol (QIAGEN). Recombinant proteins were analyzed by SDS-PAGE-SDS analysis. Purified proteins were dialyzed overnight against buffer used in semipermeabilization assays and stored at –80°C in 50 μl aliquots.

Immunoprecipitation
Transfected cells were lysed as described above, and the proteins of interest were immunoprecipitated as described previously (Bonifacino et al., 2004).

Immunofluorescence microscopy and Western blot
The following antibodies were used: anti FLAG (Sigma-Aldrich), HA (BabCO), GST (Amersham Biosciences), GM130, and TGN38 (BD Biosciences). Anti- TGN46 antibody was a gift from S. Ponnambalam (University of Leeds, Leeds, UK). Anti-phospho-serine (916) PKCμ/PKD, and phospho-serine (744–748) PKCμ/PKD were from Cell Signaling. Anti-phospho-threonine (655) PKCη, and phospho-serine (729) PKCε were from Biosource International.

Transfected cells on coverslips were fixed for 10 min in 4% formaldehyde in PBS, blocked 15 min with a solution containing 2.5% of horse serum, 0.02% sodium azide, and 0.1% Tween-20 (blocking solution). Appropriate antibodies diluted in blocking buffer were added, and cells were incubated for 30 min. After washing twice with PBS/0.1% Tween-20, 1:500 dilutions in blocking buffer of the selected fluorescent goat antibody (Jackson ImmunoResearch Laboratories) were added for another 30 min incubation. The cells were washed twice again with PBS Tween-20, the first wash in presence of 1:10,000 Hoechst 33342 (Molecular Probes) to stain DNA, and mounted on slides with gelvatol (140 mM NaCl, 10 mM KH_2PO_4/Na_2HPO_4, 25 g polyvinyl alcohol, 50 ml glycerol, 6.74 g 1,4-diazabicyclo(2,2,2) octane (DABCO), pH 8.6, for 200 ml). The entire procedure was done at room temperature. Cells were visualized with a Nikon Microphot-FXA microscope. All the slides were observed with a 60× objective, and the pictures were taken with a DP30 monochrome digital camera (Olympus). The images were analized on a Windows PC with the software MagnaFIRE 2.1 (Optronics). The filters used were B2A (for Texas-red staining) and B2E (for Cy2 staining and GFP) (Nikon).

Western blots were prepared following the manufacturer's protocol for Phospho-PKD/PKCμ (Ser744/748) (Cell Signaling). Secondary goat HRP–conjugated antibodies were from Jackson ImmunoResearch Laboratories. The chemiluminescence reagent used to develop the blots was from PerkinElmer, and the imaging film, X-Omat Blue XB-1, was from Eastman Kodak Co.

All the densitometric scans were done using the NIH Image 1.62 software.

Cell permeabilization
Cells were permeabilized as described previously (Acharya et al., 1998). 10 nM of βγ complexes (β1γ2, β3γ2, β1γ2C68S, β3γ2C68S, and β4γ2) were used for each coverslip with semipermeabilized cells.

Transport assays
HeLa cells were transfected with a plasmid codifying the tsO45 mutant VSV–G protein with a GFP tag. 5 h after transfection, cells were incubated overnight at 40°C to allow accumulation of the G protein in the ER. They were incubated for 2 h at 20°C, allowing the G protein to be transported from ER to Golgi. 10 μg/ml cycloheximide (Calbiochem) was added during the last hour of incubation. The cells were incubated for different times at 32°C, and prepared for immunofluorescence microscopy, to quantitate localization of VSV–G protein at the cell surface.

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