Cell, Vol. 104, 409–420, February 9, 2001, Copyright ©2001 by Cell Press

Protein Kinase D Regulates the Fission of Cell Surface Destined Transport Carriers from the Trans-Golgi Network

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Summary

When a kinase inactive form of Protein Kinase D (PKD-K618N) was expressed in HeLa cells, it localized to the trans-Golgi network (TGN) and caused extensive tubulation. Cargo that was destined for the plasma membrane was found in PKD-K618N-containing tubes but the tubes did not detach from the TGN. As a result, the transfer of cargo from TGN to the plasma membrane was inhibited. We have also demonstrated the formation and subsequent detachment of cargo-containing tubes from the TGN in cells stably expressing low levels of PKD-K618N. Our results suggest that PKD regulates the fission from the TGN of transport carriers that are en route to the cell surface.

Introduction

A mechanism must exist to balance the organization of Golgi stacks during protein transport. Otherwise, the Golgi stacks risk the possibility of conversion into transport carriers. We have identified a marine sponge metabolite called ilimaquinone (IQ), which when added to vertebrate cells converts Golgi stacks into small vesicles (Takizawa et al., 1993). Simply put, IQ tips the balance in the direction of uncontrolled membrane fission and the stacks are converted into small vesicles. We reasoned that analysis of IQ-mediated reactions should reveal the mechanisms regulating membrane fission. Furthermore, we suggested that the fission process activated by IQ treatment is the same as that utilized to form transport carriers from Golgi stacks under physiological conditions (Takizawa et al., 1993; Jamora et al., 1997, 1999). The IQ-mediated Golgi vesiculation was reconstituted in permeabilized cells and found to involve the trimeric G protein subunit $\beta\gamma$ (Jamora et al., 1997). Further analysis revealed that $\beta\gamma$ binds to the pleckstrin homology (PH) domain of a serine/threonine kinase called protein kinase D (PKD) and that this interaction causes vesiculation of Golgi stacks (Jamora et al., 1999). In our studies, we have used a purified preparation of $\beta\gamma$ subunits containing all isoforms from bovine brains. The exact isoform of $\beta\gamma$ that causes Golgi vesiculation therefore is not known. It is interesting to note that there are two additional isoforms of PKD and these are called PKD2 and PKDν, respectively (Hayashi et al., 1999; Sturany et al., 2000). While PKD is localized to the Golgi apparatus (in addition to the cytosol and plasma membrane), the precise intracellular locales of PKD2 and PKDν are not known. However, all of these kinases have similar structural features (two cysteine rich domains, a pleckstrin homology domain, and a catalytic domain). G$\beta\gamma$ is known to activate a number of intracellular components including the phospholipid-dependent enzymes such as phosphoinositide 3 kinases (Maier et al., 2000). Additionally, the mixture of G$\beta\gamma$ subunits used in our assay most likely activate all three (and other hitherto unidentified) isoforms of PKD and thus catalyze the en masse vesiculation of Golgi stacks. Therefore, while these studies have revealed the involvement of the trimeric G protein subunit $\beta\gamma$ and PKDs in regulating membrane fission, the specificity of these components and the order in which they act along the pathway regulating membrane fission is not clear. The stacks of Golgi cisternae communicate via transport carriers with the endoplasmic reticulum (ER), endosomes, and the plasma membrane. Is the formation of transport carriers destined for these compartments regulated by different isoforms of PKD? In order thus to understand the specificity of these kinases and their involvement in the bona fide process of intracellular protein transport we have now examined the role of PKD by using a kinase inactive form of PKD (PKD-K618N).

We have found that expression of PKD-K618N causes tubulation of the TGN and the cargo specifically destined to the plasma membrane is found in the TGN-derived, PKD-K618N-containing tubes. However, the tubes do not detach from the TGN. As a result, transfer of cargo from the TGN to the plasma membrane is inhibited. Under these conditions, protein transport from the endoplasmic reticulum (ER) to the late Golgi compartments, from the TGN to the endosomes, and to the ER is not affected. The PKD-K618N-containing, TGN-derived tubes are not coated with clathrin or the COP1 components.

We have generated a stable cell line of HeLa cells expressing moderate levels of PKD-K618N (HeLa$\sim$GF17). When these cells are cultured at 37°C, PKD-K618N is found on the TGN, the plasma membrane, and the cytosol. At this temperature, there is no obvious effect on the organization of the Golgi apparatus. However, when these cells are shifted to 20°C, PKD-K618N is found localized mostly on the TGN and the corresponding membranes are tubulated. Protein transport from the TGN to the cell surface is blocked under these conditions. Interestingly, upon shifting these cells to 25–37°C, the PKD-K618N-containing tubes detach from the TGN. These tubes eventually fuse with the plasma membrane and the cargo that was trapped in PKD-K618N-containing tubes is delivered to the plasma membrane.

Our findings illustrating the role of PKD in the fission

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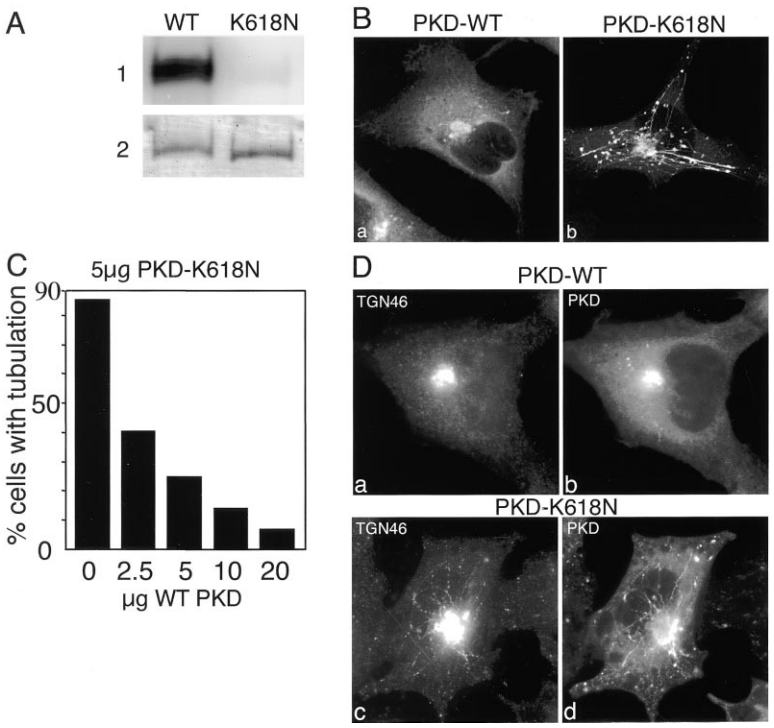

Figure 1. PKD-K618N Localizes to the Golgi Apparatus and Causes Extensive Tubulation

(A) GST-tagged wt PKD and PKD-K618N were expressed in 293T cells. The proteins were isolated by adsorption on anti-GST column from the cell lysates and incubated with 32P-ATP for 10 min at 32°C. The kinase reaction and the radioactivity associated with the respective kinases were carried out as described previously (Jamora et al., 1999). Top panel (1), autophosphorylation of PKD; bottom panel (2), coomassie blue staining to show the amounts of respective proteins used in this experiment. PKD-K618N is less than 2% active in autophosphorylation compared with the wt PKD.

(B) HeLa cells expressing GST∼PKD-K618N or wt GST∼PKD were visualized by staining with the anti-GST antibody. The wt PKD is found in the pericentriolar Golgi apparatus and diffusely dispersed in the cytoplasm (a). PKD-K618N is found predominantly in the pericentriolar Golgi apparatus and the Golgi-associated tubes (b).

(C) HeLa cells were cotransfected with 5 μg of PKD-K618N and increasing amounts of wt PKD. The cells were visualized after 72 hr by fluorescence microscopy using anti-GST antibody. Coexpression of wt PKD prevents the PKD-K618N-mediated Golgi tubulation.

(D) HeLa cells expressing GST∼wt PKD or GST∼PKD-K618N were stained with an anti-TGN46 and anti-PKD antibody. The cells were visualized by fluorescence microscopy. Localization of TGN46 and wt GST-PKD (a and b) in HeLa cells expressing wt PKD; localization of TGN46 and PKD-K618N in GST∼PKD-K618N expressing HeLa cells (c and d) is shown.

of transport carriers specifically from the TGN are described below.

Results

The PKD Inactive Kinase (PKD-K618N) Localizes to the Trans-Golgi Network and Promotes Tubulation

PKD is tightly associated with the Golgi membranes and additionally present in the cytosol (Prestle et al., 1996; Jamora et al., 1999). The levels of PKD are low in HeLa cells compared with other cell types (Johannes et al., 1994; Jamora and Malhotra, unpublished). We reasoned that expressing a kinase inactive form of PKD would be dominant over the low levels of endogenous PKD in these cells. These cells would therefore provide an opportunity to test the in vivo significance of PKD. A mutant form of PKD was generated by replacing Lysine 618 with Asparagine (K618N) in the ATP binding site of its catalytic domain. This form cannot bind ATP and is therefore inactive as a protein kinase (Zugaza et al., 1996; Waldron et al., 1999). To confirm this, the GST-conjugated wild-type (wt) and PKD-K618N were expressed in 293T cells. The proteins were isolated by adsorption on GST∼ beads and tested for their ability to undergo autophosphorylation (a test for the kinase activity of PKD). PKD-K618N is less than 2% active in autophosphorylation compared with the wt PKD (Figure 1A).

HeLa cells transfected with either the GST∼PKD-K618N or the wt GST∼PKD were stained with anti-GST antibodies. GST∼PKD localizes to a pericentriolar region, the plasma membrane, and is additionally found diffusely dispersed in the cytosol (Figure 1B). This confirms our previous findings and those of others that a significant fraction of PKD localizes to the Golgi apparatus (Prestle et al., 1996; Jamora et al., 1999). Unlike wt PKD, PKD-K618N is almost entirely localized to the Golgi apparatus and on the tubes emanating from the Golgi apparatus (Figure 1B). The transfection efficiency in these experiments is between 50% and 70% of cells and the Golgi apparatus tubulated in 40%, 60%, and 85% of the transfected cells after 24 hr, 48 hr, and 72 hr, respectively, of transfection. For the experiment described below, 200 cells on duplicate coverslips were counted to document the organization of the Golgi apparatus but, for the sake of convenience, only one cell/figure is shown.

To test whether the tubulation of the Golgi apparatus is specifically due to PKD-K618N expression, HeLa cells were cotransfected with 5 μg of PKD-K618N and increasing amounts of the wt PKD. Visualization of the cells by fluorescence microscopy using anti-GST antibody revealed that wt PKD effectively competes with the tubulation activity of the kinase inactive PKD-K618N (Figure 1C). These findings illustrate that there is a strong connection between the kinase activity of PKD and the Golgi tubulation.

The wt PKD and PKD-K618N show best colocalization with TGN46 of the TGN, compared with other Golgi-specific markers such as Giantin, GRASP 65, GM130, and Mannosidase II (Figure 1D and data not shown). Interestingly, TGN46 is found in the PKD-K618N-containing tubes. TGN46 is ordinarily transported from TGN to the plasma membrane and then back to the TGN via

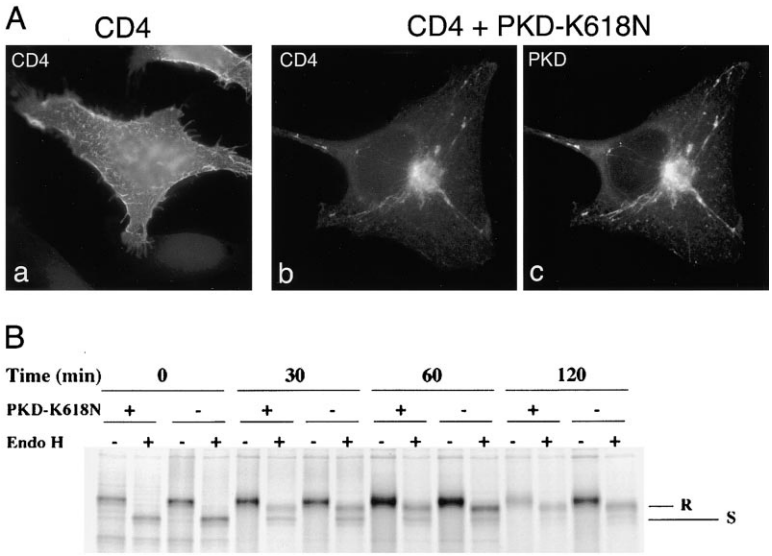

Figure 2. PKD-K618N Expression Inhibits Protein Transport from Golgi Apparatus to the Cell Surface

(A) HeLa cells were cotransfected with GST∼PKD-K618N and CD4. CD4 is made in the ER and transported to the cell surface via the Golgi apparatus. The cells were costained with anti-GST antibody and OKT4 antibody to visualize GST∼PKD-K618N and CD4, respectively. PKD-K618N is found on the Golgi apparatus and the associated tubes (c). CD4 is retained in the Golgi apparatus and the associated tubes and not found at the cell surface (b). In cells expressing CD4 only, as expected, the protein is found at the cell surface (a).

(B) CD4 has two oligosaccharide chains, which undergo core glycosylation in the ER. The ER-specific form of CD4 is endo H sensitive. Upon reaching the Golgi apparatus, one of its oligosaccharide chains undergoes maturation by the Golgi-specific glycosyltransferases. The medial and post medial cisternae form of CD4 is resistant to digestion with endo H. In control cells (expressing CD4 only), CD4 becomes fully endo H resistant 2 hr after pulse labeling of the newly synthesized proteins. CD4 in cells expressing both CD4 and PKD-K618N also becomes fully endo H resistant after 2 hr of the pulse labeling. Thus PKD-K618N expression does not affect the transport of CD4 from ER to the medial cisternae of the Golgi apparatus. R and S denote the endo H resistant and sensitive forms of the oligosaccharide chains, respectively.

endosomes (Banting and Ponnambalam, 1997). Thus, PKD-K618N expression in HeLa cells causes its localization predominantly to the TGN. Under these conditions, the TGN is tubulated and the tubes contain the recycling protein TGN46.

Protein Transport from the Trans-Golgi Network to the Cell Surface Is Inhibited in Cells Expressing PKD-K618N

HeLa cells were cotransfected with PKD-K618N and CD4. The latter is an integral membrane protein that is made in the ER and transported to the cell surface via Golgi apparatus (Nilsson et al., 1989). The transfected cells were visualized by fluorescence microscopy using antibodies against the respective proteins. In PKD-K618N expressing cells, CD4 is contained in the Golgi apparatus and in Golgi-associated PKD-K618N-containing tubes. However, CD4 does not reach the cell surface under these conditions (Figure 2Ab). In control cells (i.e., not expressing PKD-K618N), CD4 is found at the cell surface (Figure 2Aa).

Is CD4 retained in the *cis*-Golgi cisterna or does it travel across the Golgi stack and fail to exit from the TGN? To address this issue, the following experiment was carried out. CD4 is core glycosylated at two sites in the ER. The ER-specific oligosaccharide chains of CD4 are sensitive to digestion with the enzyme endoglycosidase H (endo H). During its transport along the Golgi stacks, one of its oligosaccharide chains undergoes further processing by the Golgi-specific glycosyltransferases. This particular oligosaccharide chain of CD4 in the medial and post-medial Golgi cisternae, therefore, becomes resistant to digestion with endo H. This change in sensitivity to endo H treatment, therefore, provides a useful assay to map the location of CD4 in the Golgi stacks. HeLa cells were cotransfected with PKD-K618N and CD4 or with CD4 alone. After 72 hr, the cells were labeled with ^{35}S-methionine for 15 min at 37°C and subsequently incubated with medium containing unlabeled methionine. CD4 was immunoprecipitated after 0, 30, 60, and 120 min from these cells. One half of the precipitate was incubated with endo H and the other half with buffer alone. Endo H treated and untreated CD4 samples were analyzed by SDS-PAGE/autoradiography. Our results show that the newly synthesized CD4 in cells expressing PKD-K618N acquires resistance to endo H with the same kinetics as in cells not expressing PKD-K618N (Figure 2B). Thus, CD4 is transported across the Golgi stacks to at least the medial Golgi cisternae in cells expressing PKD-K618N.

PKD-K618N Expression Neither Inhibits Protein Transport from TGN to the Endosomes nor Affects the Organization of ER, the Transitional ER, and the Intermediate Compartment

HeLa cells were cotransfected with PKD-K618N and H2-M. H2-M is a resident protein of endosomes/lysosomes. It is made in the ER and transported to endosomes via the Golgi apparatus (Karlsson et al., 1994). HeLa cells expressing PKD-K618N and H2-M were stained with the respective antibodies and visualized by fluorescence microscopy. In cells expressing H2-M alone, it is localized to punctate structures (Figure 3Aa). These punctate structures are the endosomes/lysosomes as shown previously (Karlsson et al., 1994). Coexpression of PKD-K618N does not affect the transport of H2-M to similar punctate structures (Figure 3Ab). Additionally, there is no accumulation of H2-M in ER and the Golgi apparatus in PKD-K618N expressing cells. A defect in transport of H2-M to endosomes would cause its accumulation in the compartments en route. However, one could argue that the endosomal localized H2-M is a fraction of the protein that reached there prior to the expression of PKD-K618N at threshold levels

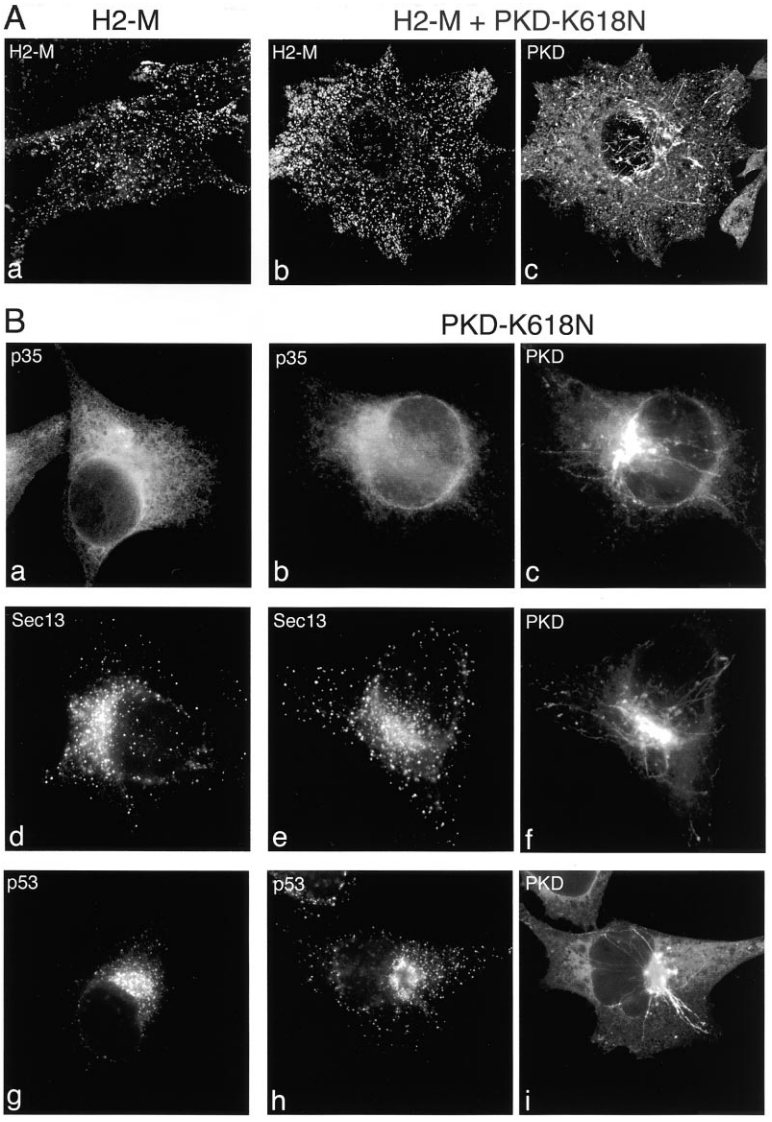

Figure 3. PKD-K618N Expression Neither Blocks Protein Transport from the Golgi Apparatus to the Endosomes nor Affects the Organization of Other Compartments of the Secretory Pathway

(A) HeLa cells were cotransfected with PKD-K618N and the endosomal resident protein H2-M. These proteins were visualized by fluorescence microscopy with specific antibodies to PKD and H2-M, respectively. H2-M is found in small punctate structures (b) in cells expressing PKD-K618N, which are similar to control cells expressing H2-M only (a).

(B) HeLa cells were transfected with the Invariant chain p35 only (a), cotransfected with GST∼PKD-K618N and p35 (b and c) or with GST-PKD-K618N only (e, f, h, and i). The cells were stained with antibodies to p35 (an ER marker in [a]); p35 and GST (b and c); Sec13p (d); Sec13p and GST (e and f); ERGIC53 (g); ERGIC53 and GST (h and i). Expression of PKD-K618N does not perturb the organization nor does it cause tubulation of the compartments containing p35 (ER marker), Sec13 (transitional ER marker), and p53 (ERGIC 53 or the intermediate compartment marker).

necessary for inhibiting this transport step. To address this potential concern, the following experiment was carried out. PKD-K618N was cotransfected with the endosomal marker H2-M. H2-M was under the tetracycline-inducible promoter. H2-M was induced 16 hr after PKD-K618N transfection to ensure that tubulation had occurred before H2-M reached the Golgi apparatus. After 72 hr, the cells were visualized by fluorescence microscopy using antibodies to the proteins H2-M and PKD, respectively. Under these experimental conditions, the Golgi membranes were tubulated and H2-M was found in the endosomes (data not shown). Thus, PKD-K618N expression does not affect protein transport from ER to the endosomes via TGN.

Expression of PKD-K618N does not affect the recycling of proteins from Golgi apparatus to the intermediate compartment and to the ER. This is evident from our findings that the organization of ERGIC53 (p53), which cycles between the intermediate compartment and the early Golgi cisternae (cis/medial), is unaffected in cells expressing PKD-K618N (Figure 3B). In addition, the localization of the KDEL receptor, which cycles between ER and the Golgi apparatus via the intermediate compartment, is unaffected in cells expressing PKD-K618N (data not shown).

HeLa cells were cotransfected with GST∼PKD-K618N and the Invariant chain p35 (an ER marker; Kuwana et al., 1998), or transfected with PKD-K618N alone. The cells were stained with anti-GST antibody to visualize PKD-K618N and antibodies to the compartment-specific markers such as anti-p35 antibody, Sec13p antibody (a marker for the transitional ER; Hammond and Glick, 2000), and ERGIC53 antibody (p53, an intermediate compartment-specific protein; Schindler et al., 1993). The cells were visualized by fluorescence microscopy. Our results show that expression of PKD-K618N has no obvious effect on the organization of compartments of the secretory pathway preceding the Golgi apparatus (Figure 3B).

PKD-K618N expression does not affect the organization of early and late endosomes as monitored by fluorescence microscopy using anti-EEA1 antibody (for

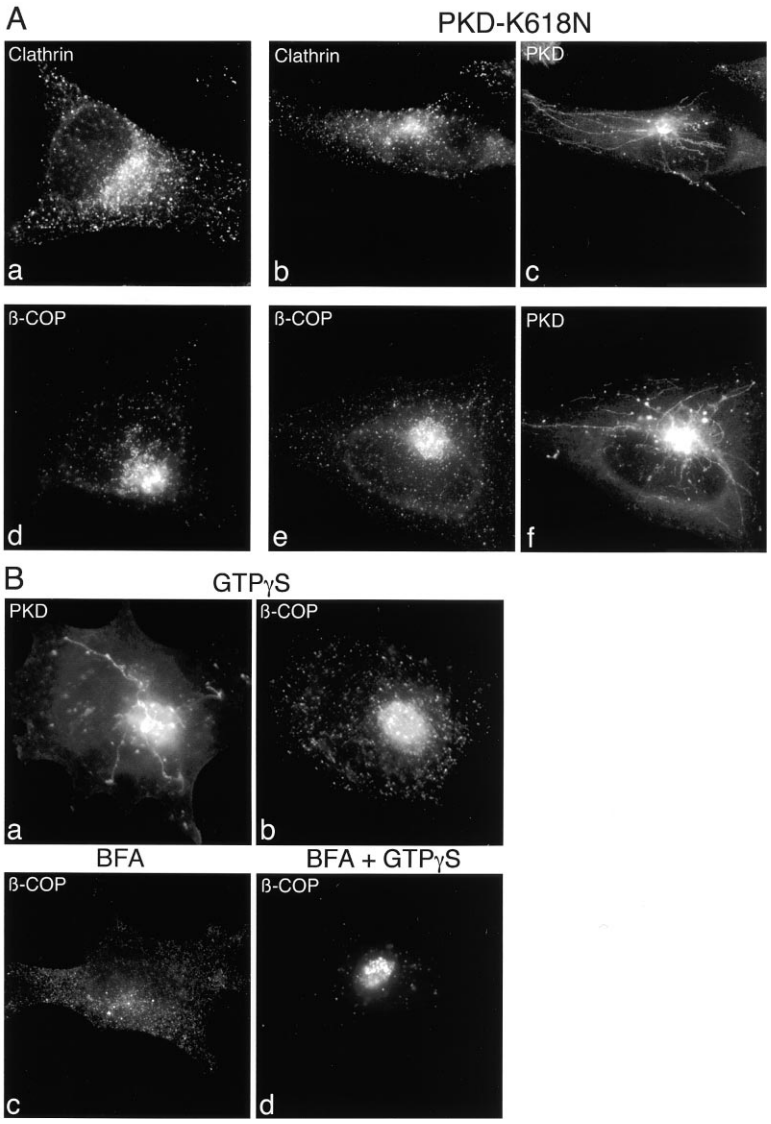

Figure 4. The Tubulated Golgi Membranes Do Not Contain COP1 and Clathrin Coats

(A) HeLa cells were transfected with GST∼PKD-K618N and the cells double labeled with antibodies to the COP1-specific subunit β-COP or clathrin. The normal localization of clathrin and β-COP in nontransfected HeLa cells is shown in (a) and (d). In cells expressing PKD-K618N, the clathrin (b) and β-COP (e) staining is evident in the core of the Golgi apparatus. The PKD-K618N-containing tubes in these cells (c and f) do not contain clathrin or the β-COP.

(B) HeLa cells expressing GFP∼PKD-K618N were permeabilized and incubated with GTPγS (a and b). The cells were stained with the anti-β-COP antibody. PKD-K618N-coated tubes do not contain β-COP (b). HeLa cells were permeabilized and treated with BFA in the absence (c) or the presence of GTPγS (d). GTPγS prevents the BFA-mediated dissociation of β-COP (d). Thus, GTPγS is functional in promoting COP1 coating of the Golgi membranes under our experimental conditions.

early endosomes) and anti-LAMP1 antibody (for late endosomes and lysosomes) (data not shown).

Taken together, these results provide very strong evidence for the effects of PKD-K618N expression on protein transport and tubulation, specifically to its locale of the TGN.

PKD-K618N-Induced TGN Tubes Do Not Contain β-COP or Clathrin

The cytosolic proteins ARF and COP1 coats are required to form the COP1 vesicles from Golgi cisternae (Orci et al., 1993). Treatment with Brefeldin A (BFA) causes dissociation of COP1 coats from the Golgi apparatus. The Golgi membranes tubulate and eventually fuse with the ER (Lippincott-schwartz et al., 1989). PKD-K618N expression causes tubulation of the TGN, but the tubes do not fuse with the target membrane. We, therefore, sought to test whether the tubes generated by PKD-K618N expression are COP1 coated. HeLa cells expressing PKD-K618N were stained with an anti-β COP antibody and visualized by fluorescence microscopy. β-COP is found on the Golgi apparatus but not the tubes containing PKD-K618N (Figure 4A). The localization of β-COP in control (nontransfected) cells to the Golgi apparatus and punctate structures (presumably, the VTC's) is shown in Figure 4A for comparison. Therefore, unlike BFA treatment, the expression of PKD-K618N does not result in the dissociation of COP1 coats from Golgi apparatus.

While β-COP is required to form COP1-coated vesicles, clathrin is needed for the formation of clathrin-coated vesicles from the TGN. HeLa cells expressing PKD-K618N and costained with the anti-clathrin antibody revealed that clathrin colocalizes with the core of PKD-K618N-containing TGN but not the tubes (Figure 4A).

Our results show that PKD-K618N-containing tubes originate in the TGN and lack clathrin and COP1 coats. This fits well with our findings that expression of PKD-K618N does not affect protein transport from TGN to the endosomes (which is mediated by clathrin-coated vesicles) and to the ER (which is mediated by COP1 vesicles). However, we reasoned that coats might dissociate

once the PKD-K618N-containing tubes had reached a certain dimension. This is a reasonable argument since it is known that COP1 coats dissociate rapidly after the formation of COP1 vesicles (Orci et al., 1989). To address this issue, we made use of the finding that treatment with the nonhydrolyzable analog of GTP, namely GTPγS, prevents uncoating (Melancon et al., 1987; Malhotra et al., 1989). HeLa cells were transfected with GFP∼PKD-K618N and two days after the transfection, cells were permeabilized with digitonin and incubated with 100 μM GTPγS for 30 min at 32°C. A control consisted of GFP∼PKD-K618N-expressing cells that were permeabilized and incubated with the buffer used for dissolving GTPγS. The cells were visualized by fluorescence microscopy with anti-βCOP antibody. While the core of the Golgi apparatus contains β-COP, the PKD-K618N-containing tubes are devoid of β-COP (Figure 4B). Thus, under conditions that promote binding of COP1 coats to the Golgi membranes, the PKD-K618N-containing Golgi tubes are uncoated and remain attached to the TGN. To test whether GTPγS is effective in coating the Golgi membranes with COP1 coats, the following experiment was carried out. As mentioned above, one of the earliest effects of Brefeldin A (BFA) treatment is the loss of COP1 coats from the Golgi apparatus. However, treatment with GTPγS inhibits the BFA-mediated shedding of COP1 coats (Donaldson et al., 1992). HeLa cells were permeabilized, incubated with GTPγS, and then treated with BFA. The cells were visualized with the anti-β-COP antibody. In control cells (not treated with GTPγS), β-COP dissociates from the Golgi apparatus by BFA treatment (Figure 4B). In cells treated with GTPγS, βCOP remains attached to the Golgi membranes in the presence of BFA. Therefore, GTPγS is effective in promoting the binding (and retention) of COP1 coats under our experimental conditions. These findings lend further support to our proposal that PKD-K618N-containing tubes are derived from the TGN, are en route to the plasma membrane, and do not contain COP1 and clathrin coats.

The Accumulation and Consumption of PKD-K618N-Containing, TGN-Derived Tubes

The data presented above suggest that a domain of the TGN tubulates when the kinase activity of PKD is compromised. The tubes contain cargo destined for the cell surface but fail to dissociate. But are these tubes the transport carriers in the TGN to the cell surface step of the secretory pathway? If so, we should be able to find conditions in which these tubes detach from the TGN and fuse with the plasma membrane. We have designed an experimental strategy to address precisely this issue. We have generated a stable line of HeLa cells that express moderate levels of GST∼FLAG∼PKD-K618N. These cells, called HeLa∼GF17, express full-length GST∼FLAG-PKD-K618N (data not shown). Fluorescence microscopic analysis with anti-GST antibody of these cells reveals that a fraction of PKD-K618N is localized to the Golgi apparatus. In addition, PKD-K618N is found at the cell surface and the cytosol. These cells show no obvious defect in the organization of the Golgi apparatus at 37°C (Figure 5). When these cells are maintained at 20°C for 2–3 hr, the PKD-K618N-containing TGN undergo extensive tubulation in greater than 90% of the cells (Figure 5). While PKD-K618N-containing tubes attached to the TGN are evident in these cells, the cells are also found to contain short PKD-K618N-containing tubules detached from the TGN. These short fragmented tubules may be the result of incomplete inhibition of the fission process during the shift from 37°C to 20°C. In control cells, i.e., cells not expressing PKD-K618N, tubulation of the TGN was observed (by staining with the anti-TGN46 antibody) in fewer than 10% of the cells (data not shown). Thus, tubulation of the TGN at 20°C is a quantitative effect of PKD-K618N expression. In HeLa∼GF17 cells, the tubes remain attached to the Golgi apparatus even after an additional 3 hr of incubation at 20°C in 60% of the cells. But if, after 2 hr of incubation at 20°C, the cells are shifted to 25°C (or to a higher temperature of 37°C) for 30 min, the tubes are found detached from the Golgi apparatus in 70% of the cells. After 3 hr of incubation at higher temperatures (25–37°C), the Golgi membranes appear normal, i.e., without any tubulation, in greater than 70% of the cells (Figure 5). Thus, there is a temperature-dependent formation and dissociation of the PKD-K618N-containing tubes in these cells. PKD cycles between the cytosol and the Golgi apparatus. A simple explanation for our findings is that there is a temperature-sensitive defect in the recycling of PKD. In HeLa∼GF17 cells, PKD-K618N binds to the Golgi apparatus and then dissociates at 37°C. However, because of the low levels of PKD-K618N expression, there is little noticeable effect on the organization of TGN at 37°C. When the cells are shifted to 20°C, PKD-K618N binds to the TGN but fails to dissociate. Thus, it accumulates on the Golgi membranes and acts as dominant negative over the low levels of endogenous kinase. Upon shift to 37°C, the cycling of PKD-K618N is restored, the tubes dissociate, and the Golgi apparatus returns to its normal organization. This temperature-dependent accumulation and subsequent detachment of the PKD-K618N tubes illustrates that these tubes are not a dead end product but a kinetic intermediate in the TGN to the cell surface step of the transport process.

The PKD-K618N-Containing Tubes Are Intermediates in Protein Transport from TGN to the Cell Surface

Do PKD-K618N-containing tubes carry cargo from TGN to the cell surface in HeLa∼GF17 cells? HeLa∼GF17 cells were infected with the tsO45 strain of vesicular stomatitis virus (VSV). At the nonpermissive temperature of 40°C, the VSV-G protein is made and retained in the endoplasmic reticulum. This is because of a temperature-sensitive defect in the folding of VSV-G protein. Upon shifting the cells to 20°C, the VSV-G protein folds and is transported along the secretory pathway to a late Golgi compartment (Griffiths et al., 1985). However, protein transport from the late Golgi compartment to the cell surface is blocked under these conditions. Shifting cells to a temperature of 25–32°C restores protein transport from the late Golgi compartments to the cell surface. The mechanism by which transport from the TGN is blocked at 20°C is not known but it provides a useful means to monitor protein transport along this step. We carried out this experiment in HeLa∼GF17 cells. Newly

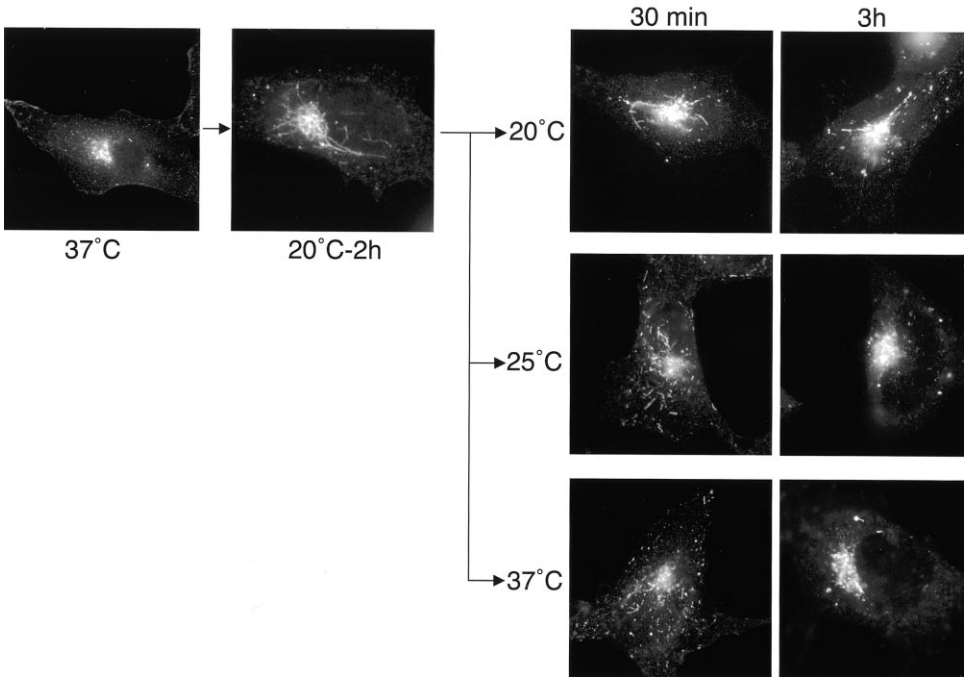

Figure 5. The Temperature-Sensitive Accumulation and Detachment of PKD-K618N-Containing Tubes

HeLa cells stably expressing GST∼FLAG∼PKD-K618N (Hela∼GF17) were incubated at 20°C for 2 hr. The cells were then kept at 20°C or shifted to 25°C and 37°C. The cells were stained with anti-GST antibody after 30 min and 3 hr of incubation at the respective temperatures. Control cells incubated at 37°C show the localization of PKD-K618N to the Golgi apparatus, the cytosol, and the plasma membrane. Shifting the cells to 20°C causes extensive tubulation of the Golgi apparatus and the tubes are PKD-K618N coated. Within 30 min of incubation at 25°C and 37°C, the PKD-K618N-containing short tubes are detached from the core of PKD-K618N staining TGN. After 3 hr of incubation at 25°C and 37°C, the Golgi apparatus appears almost normal. However, in cells kept at 20°C, the PKD-K618N-containing tubes remain attached to the TGN.

synthesized VSV-G protein was arrested in the late Golgi compartment by the 20°C temperature block for 2 hr in the presence of cycloheximide. The cells were shifted to 25°C and visualized by fluorescence microscopy using anti-VSV-G and GST antibodies. Immediately upon shift from 20°C to the 25°C (0 min time point, Figure 6), the VSV-G protein is found in PKD-K618N tubes attached to the Golgi apparatus. At 20–30 min of incubation at 25°C, the PKD-K618N and VSV-G protein-containing tubes are found detached from the Golgi apparatus. After 60 min of incubation at 25°C, the PKD-K618N and VSV-G protein-containing small membranes are found closer to the plasma membrane. In fact, at this time in about 20% of the cells, the VSV-G protein can be detected at the cell surface. At 90 min, in greater than 70% of the cells, the VSV-G protein is found at the cell surface. At this time point, the number of PKD-K618N tubes is markedly reduced and PKD relocates to the Golgi apparatus, cytosol, and the plasma membrane (Figure 6).

As mentioned above, TGN46 cycles between TGN and the endosomes via the plasma membrane. In HeLa cells expressing PKD-K618N by transient transfection, TGN46 is trapped in PKD-K618N-containing tubes (Figure 1D). In HeLa∼GF17 cells kept at 37°C, the TGN46 colocalizes with the Golgi-associated pool of PKD-K618N and the Golgi membranes appear normal, i.e., without any obvious tubes (Figure 7). HeLa∼GF17 cells were shifted to 20°C for 2 hr and visualized by fluorescence microscopy using anti-TGN46 and anti-PKD antibodies. Under these conditions, the TGN46 is found in PKD-K618N tubes (Figure 7). The tubes are attached to the TGN at this stage of the incubation. After 30 min of incubation at 25–37°C, the PKD-K618N- and TGN46-containing membranes are found detached from the Golgi apparatus (data not shown). Taken together, these findings illustrate that PKD-K618N-containing tubes are the carriers of cargo such as VSV-G protein and TGN46 from TGN to the cell surface in HeLa∼GF17 cells.

Discussion

The identity of transport carriers from TGN to the cell surface (other than the secretory storage granules) and the mechanism by which they form is largely unknown. This is in contrast to the well-characterized COP1, COPII, and clathrin-coated vesicles. In all of these latter cases, their formation is shown to require a small molecular weight GTPase and a complex of cytosolic proteins called the coat proteins (COP1, COPII, and clathrin coats). In contrast, we have found that when the kinase activity of PKD is compromised by overexpressing an inactive kinase (PKD-K618N), the cargo contained in the TGN is captured into tubes but the tubes do not detach. By using a stable cell line expressing low levels of PKD-K618N, we have shown that PKD-K618N-containing tubes are transport intermediates from TGN to the plasma membrane.

We have reconstituted the accumulation and subse-

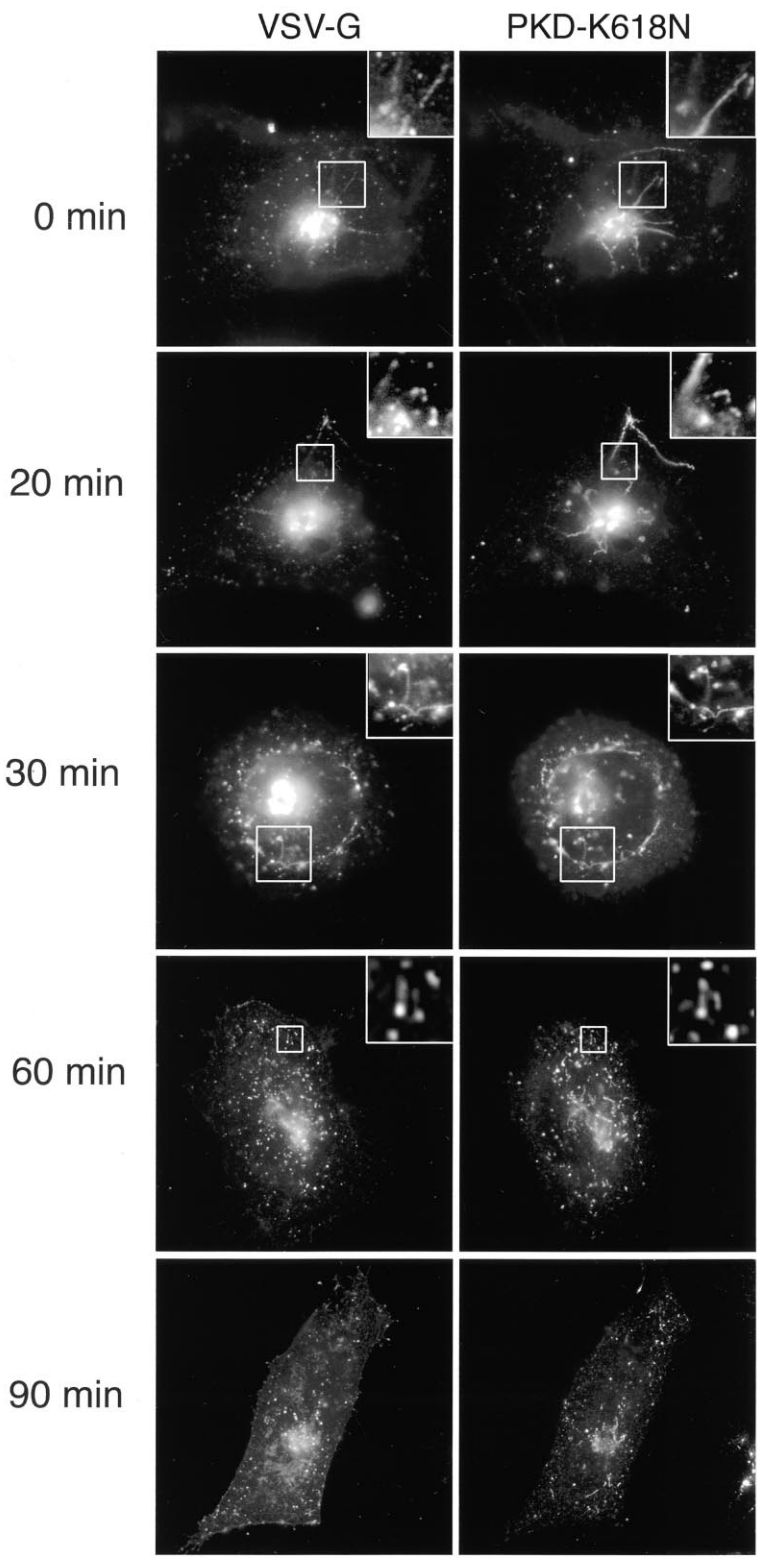

Figure 6. A Time-Dependent Detachment and Consumption of Cargo-Containing PKD-K618N Tubes in HeLa∼GF17

HeLa∼GF17 cells were infected with the tsO45 strain of VSV at 32°C. The cells were shifted to 40°C for 2.5 hr, to 32°C for 25 min, and then to 20°C for 2 hr. The cells were then transferred to 25°C. At the time points shown, the cells were fixed and stained with the anti-VSV-G and GST antibody to visualize VSV-G protein and PKD-K618N, respectively. At the onset of the reaction at 25°C, VSV-G protein and PKD-K618N-containing tubes are found attached to the TGN. This is also evident after 20 min of incubation. However, by 30 min of incubation at 25°C, the two proteins are contained in small tubes and punctate elements that are not attached to the Golgi apparatus. By 90 min, VSV-G protein is detected at the cell surface in greater than 70% of the cells. At this time point PKD-K618N relocates to the Golgi and the cytosol. The boxed areas are examples showing colocalization of VSV-G and PKD-K618N.

quent consumption of the PKD-K618N-containing tubes in a temperature-dependent manner. It has been known for a number of years that protein transport from the late Golgi compartments to the cell surface is blocked at 20°C (Griffiths et al., 1985). The mechanism by which the 20°C incubation inhibits protein exit from the TGN is not known. Under these conditions, the cargo destined to the cell surface remains in the TGN but there is no detectable tubulation. However, in cells expressing PKD-K618N (HeLa∼GF17), the TGN undergoes exten-

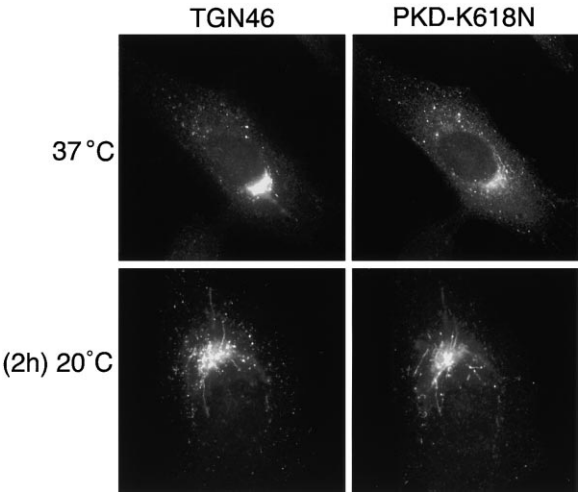

Figure 7. The Recycling Protein TGN46 Is Contained in the TGN-Derived PKD-K618N Tubes in HeLa~GF17 Cells

Hela~GF17 cells were incubated at 37°C and then shifted to 20°C for 2 hr. The cells were stained with anti-GST and TGN46 antibodies. TGN46 and PKD-K618N are found in tubes attached to the Golgi apparatus at 20°C.

sive tubulation. The tubes containing cargo destined for the cell surface accumulate as a result of PKD-K619N expression in these cells at 20°C. Upon shifting the cells to 25°C, the tubes detach from TGN and eventually fuse with the cell surface. A likely possibility is that the 20°C incubation inhibits recycling of PKD-K618N and, over a time period of 2–3 hr, causes its accumulation on the TGN. This creates a situation in which transport carriers form but fail to undergo fission, consequently accumulating as tubes attached to the TGN. When these cells are transferred to higher temperatures (25–37°C), the cycling of PKD-K618N between the TGN and the cytosol is restored, the tubes detach from the TGN and eventually fuse with the cell surface, thus restoring protein trafficking between these two compartments. These findings suggest a pivotal role of PKD in regulating the fission (detachment) of cargo-containing tubular elements from TGN to the cell surface.

PKD-Mediated Membrane Fission Is Highly Compartmentalized

PKD was identified as a component of the Golgi vesiculation process that is triggered by treatment with ilimaquinone (Jamora et al., 1999). We have shown that IQ treatment activates a trimeric G protein on the Golgi apparatus. Sequestering $G\beta\gamma$ with $G\alpha\sim GDP$ inhibits IQ-mediated Golgi fragmentation. Adding purified bovine brain $\beta\gamma$ subunits without treatment with IQ causes fragmentation of the Golgi apparatus. These results suggest that $\beta\gamma$ subunits regulate the equilibrium between the Golgi apparatus as stacks of cisternae and as vesicles (Jamora et al., 1997). Further studies revealed that PKD was the downstream target of $\beta\gamma$ subunits (Jamora et al., 1999). In other words, overactivation of PKD via IQ and $\beta\gamma$ subunits converts Golgi stacks into vesicles through uncontrolled activation of the fission machinery. It, therefore, makes sense that overexpression of a kinase inactive form of PKD (PKD-K618N) causes tubulation because of a defect in membrane fission. But why does treatment with IQ and $\beta\gamma$ convert Golgi stacks into vesicles whereas PKD-K618N expression affects only a subset of the TGN? One possibility is that IQ and the mixture of $\beta\gamma$ used in our studies activate other components (in addition to PKD, PKD2, and PKDν) and thus have a more global effect on the Golgi stacks. PKDwt and PKD-K618N, on the other hand, localize predominantly on the TGN and therefore affect only the TGN-specific processes.

The Partners of PKD Regulating the Formation of Transport Carriers from the TGN

The formation of PKD-K618N-containing tubes from the TGN does not seem to involve COP1 or clathrin coats. $GTP\gamma S$ treatment, which promotes coating and fission of COP1-coated membranes, has no effect on the PKD-K618N-containing tubes. The PKD-dependent, TGN-derived tubes, therefore, are either uncoated or contain a novel coat.

What is downstream of PKD? We have previously proposed that different combinations of protein and lipid kinases regulate the formation of transport carriers with distinct destinations from the Golgi apparatus (Jamora et al., 1999). The Vps15p (a protein kinase) and Vps34p (PI-3 kinase) combination is involved in the generation of clathrin-coated vesicles from TGN to the endosomes (Stack et al., 1995). We believe that phosphoinositide kinases are also involved in the PKD-mediated, but clathrin- and COP1-independent, transport carrier formation from the TGN. Support for this proposal comes from two recent findings. First, PKD is known to interact with at least two different lipid kinases. These are a type II phosphatidylinositol 4 kinase (PI-4 K) and phosphatidylinositol-4-phosphate 5 kinase (P1-4 P 5K). The PKD kinase activity is essential for this association and/or activation (Nishikawa et al., 1998). Second, PI-4 kinases are required for secretion from TGN to the cell surface in yeast (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). Identification of the PI kinases that match with PKD will help address this issue.

A Pathway for Regulating the Formation of Transport Carriers from the TGN

In summary, our findings have revealed the significance of PKD-mediated reactions in the fission of transport carriers from the TGN. A regulated fission process could, in principle, control the number and size of transport carriers budding from the TGN (and other intracellular compartments of the exocytic and endocytic pathways). For example, if the cargo is large, the process of membrane fission could be delayed until the transport carrier is large enough—a tubule for example—to encapsulate the cargo. Remember tubules as transport carriers from the Golgi to the cell surface have been visualized, but their molecular description and the mechanism of formation are not known (Cooper et al., 1990; Glick and Malhotra, 1998; Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000). From a mechanistic point of view, the difference between a small vesicle and large tube (as transport carriers) may simply be in the timing of membrane fission. But is the signal for determining

the size of the transport carriers dictated solely by the cargo? And once this decision is made, how is the reaction terminated to prevent the compartment from conversion into transport carriers? These are appealing suggestions and issues of fundamental importance, but little is known to validate their applicability during protein transport. It is clear that in order to gain a comprehensive understanding of the mechanisms regulating the balance between secretory load and the compartmental integrity, we need to identify other essential components and place the currently known players, such as βγ, PKD, and PI-kinases, in the correct order. The placement of PKD in the fission of tubes from the TGN adds a molecular component and paves the way for an understanding of regulating membrane fission in this key step of protein transport.

Experimental Procedures

Cell Culture and Transfections

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% FCS, 2 mM glutamine, and 100 U/ml penicillin and streptomycin at 100 mg/ml, and passaged every other day. For VSV transport, this media was supplemented with 25 mM Hepes buffer, pH 7.4. Transient transfections were carried out using the calcium phosphate method as previously described (Liljedahl et al., 1996); a total of 25 μg of DNA and 5 x 10^5 cells were used per transfection. The following amounts of DNA were used for transfections: Figures 1A and 1B, 25 μg wt PKD and 25 μg PKD-K618N; Figures 1Da and 1Db, 25 μg wt PKD; Figures 1Dc and 1Dd, 25 μg PKD-K618N; Figure 2Aa, 5 μg CD4 and 20 μg mock DNA; Figures 2Ab and 2Ac, 5 μg CD4 and 20 μg PKD-K618N; Figure 2B, same as in Figure 2A; Figure 3Aa, 2.5 μg H2-Mα, 2.5 μg H2-Mβ (this protein exits as a dimer of α and β subunits), and 20 μg mock DNA; Figures 3Ab and 3Ac, 2.5 μg H2-Mα, 2.5 μg H2-Mβ, and 20 μg PKD-K618N; Figure 3Ba, 5 μg p35 and 20 μg mock DNA; Figures 3Bb and 3Bc, 5 μg p35 and 20 μg PKD-K618N; Figures 3Be, 3Bf, 3Bh, and 3Bi, 25 μg PKD-K618N; Figures 4Ab, 4Ac, 4Ae, and 4Af, 25 μg PKD-K618N; Figures 4Ba and 4Bb, 25 μg GFP∼PKD-K618N.

Generation of the Stable Cell Line Expressing PKD-K618N

Hela cells were transfected with pME-Neo-GF-PKD-K618N using the calcium phosphate method. Selection with 500 μg/ml G418 (GIBCO) began two days after transfection, followed by limiting dilution to establish cell lines. One cell line, GF17, expressed full-length GST-FLAG tagged PKD-K618N analyzed by Western blotting (data not shown) and immunofluorescence microscopy using anti-GST antibody and the antibody recognizing the C-terminal portion of PKD.

Pulse Chase Experiment to Analyze the Transport of CD4

Hela cells were transfected as indicated above. 72 hr after transfection, cells were first starved for 30 min in methionine-free media and then pulse labeled in media containing 0.1 mCi ^{35}S-methionine (NEN, Life Science Products) per ml for 15 min. The cells were subsequently incubated in regular media containing methionine for 30, 60, and 120 min. At the times mentioned, the cells were lysed in RIPA buffer and CD4 protein immunoprecipitated (see below), digested with endo-H (Bio Labs, New England), and subsequently analyzed by SDS- PAGE/autoradiography.

Recombinant DNA

PKDwt sequence has been described previously (Valverde et al., 1994). The cDNA was subcloned into the Not1 site of pGMEXT3, which has a GST tag upstream of the Not1 site. The PKD kinase inactive K618N clone was constructed by changing the Lysine in the 618 position to an Aspargine by the Quickchange kit, according to the instructions of the manufacturer. pME-Neo-GF-PKD-K618N plasmid was constructed by subcloning the SalI-XbaI fragment from pGMEXT3-PKD-K618N into pME-Neo-GF vector, which has a neomycin resistance gene and GST and FLAG tandem tag for making a fusion protein. pEGFP-PKD-K618N was constructed by subcloning the SalI-SmaI fragment from pGMEXT3-PKD-K618N into pEGFP-C1 vector (Clontech).

The clones CD4 (Nilsson et al., 1989), H2-M (Karlsson et al., 1994), and p35 (Kuwana et al. 1998) were as described in the papers referenced.

Antibodies

For the PKD immunofluorescence staining, either a rabbit polyclonal anti-PKD antibody or a Goat polyclonal GST antibody was used. The anti-PKD antibody is a rabbit polyclonal antibody generated against EEREMKALSERVSIL, the C-terminal peptide of PKD. The anti-GST antibody was purchased from Amersham Pharmacia Biotech Inc. For the experiments involving CD4, either OKT4 or anti-human CD4 monoclonal antibody (R and D MAB 379) was used. Ascites fluid containing mouse monoclonal antibody OKT4 was prepared using hybridoma No. CRL-8002 from ATCC. The anti-CD107a antibody was purchased from PharMingen. The anti-EEA1 antibody was purchased from Transduction Laboratories. The anti-TGN46 antibody is a gift from Dr. Vas Ponnambalam (Leeds University), ERGIC 53 antibody from Dr. Hans-Peter Hauri (University of Basel, Switzerland). The β-COP mouse monoclonal antibody CM1A10 was a gift from Dr. Jim Rothman (Sloan-Kettering Institute). The rat monoclonal H2-M antibody was a gift from Dr. Lars Karlsson (The RW Johnson Research Institute). The anti-p35 antibody is a mouse monoclonal antibody called Pin-1 and a gift from Dr. Peter Cresswell (Yale University). The anti-Sec13 antibody is a rabbit polyclonal antibody and a gift from Dr. William Balch (The Scripps Research Institute). The anti-clathrin antibody is a rabbit polyclonal antibody and a gift from Dr. Sandra Schmidt (The Scripps Research Institute). The anti-VSV-G antibody called P5D4 (against the intracellular portion of the VSV-G) has been documented previously (Jamora et al., 1999), the anti VSV-G antibody called 8G5F11(against the extracellular portion of VSV-G) is a gift from Dr. Lyles (Wake Forest University). Secondary antibodies, goat anti-rabbit, anti-mouse, and goat anti-rat were obtained from Cappel. Donkey anti-goat, anti-rabbit, anti-mouse, and anti-sheep antibodies were obtained from Jackson Immuno Research.

Transport of VSV-G Protein in Hela Cells Stably Expressing PKD-K618N (Hela∼GF17)

The experiment was carried out as described previously (Jamora et al., 1999) with the following changes. Briefly, HeLa∼GF17 cells in 24-well plate were incubated with 0.5 ml DMEM without FCS-containing Hepes, VSV for 1 hr at 32°C, followed by additional 45 min incubation with 1.5 ml complete DMEM. The cells were then shifted to 40°C for 2.5 hr, 32°C for 25 min, 20°C for 2 hr, and finally 25°C for the times indicated.

Immunoprecipitation of PKD and the Kinase Assay by Autophosphorylation

For the experiment shown in Figure 1A, 293T cells transfected with GST-tagged PKD wt or PKD-K618N expression plasmid were lysed in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 1 mM DTT, 25 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM PMSF, 2 μg/ml Leupeptin, 0.2 μg/ml aprotinin, and 0.2 μg/ml pepstatin) for 1 hr at 4°C, followed by centrifugation at 100,000 × g for 30 min. The lysates were incubated overnight with Glutathione-sepharose (Amersham Pharmacia, Uppsala, Sweden). The precipitates were washed three times with lysis buffer and twice with elution buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% NP-40, and 1 mM DTT). PKD proteins were eluted with the elution buffer containing 20 mM reduced glutathione. 20 μl of eluted samples containing 0.5 μg PKD proteins were mixed with 10 μl 50 mM Tris pH 7.4, 30 mM $MgCl_2$, 300 μM ATP, 5 μCi γ−[32P]ATP and incubated for 10 min at 32°C. The reactions were stopped by adding sample buffer. The proteins were resolved by SDS-PAGE, the gel stained with coomassie blue, dried, and analyzed by phosphoimager.

Cell Permeabilization

The cell permeabilization assay used is a modification of Acharya et al. (1998). Briefly, HeLa cells were grown on Pronectin F-coated glass coverslips and then transfected with PKD-K618N GFP. These

cells were washed at room temperature in KHM buffer (25 mM Hepes-KOH [pH 7.2], 125 mM potassium acetate, 2.5 mM magnesium acetate), shifted to ice, washed with cold KHM, and permeabilized with 30 μg/ml digitonin in KHM. The cells were incubated at 32°C for 45 min with 100 μM GTPγS and ATP regeneration system in KHM buffer.

Acknowledgments

This manuscript is dedicated to the memory of the very young Chantal Vogelzangs. Lars Karlsson and Michael R. Jackson of the RW Johnson Pharmaceutical Research Institute, Ben Glick, John Faulkner, and members of the Malhotra lab are thanked for useful discussions. M. Liljedahl is supported by a fellowship from the Leukemia and Lymphoma Society, USA; Y. Maeda by a fellowship from the Uehara Memorial Foundation, Japan; A. Colanzi by a fellowship from the Consiglio Nazionale Delle Ricerche, Italy. J. Van Lint is supported by 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen' and 'Interuniversitiare Attractiepolen Grant P4/26.' V. Malhotra is an established investigator of the American Heart Association and the work in Malhotra's lab is supported by grants from the NIH.

Received April 6, 2000; revised January 10, 2001.

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