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Research article

Regulation of PKD1-mediated Golgi to cell surface trafficking by Gαq subunits

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Background Information. Heterotrimeric GTP-binding proteins play a key role in cell trafficking regulation. Above all, specific Gβγ subunits have been shown to be a major component of a signal transduction pathway, which also involves phospholipases C (PLC), protein kinases C (PKC) and D (PKD), whose main function is to regulate transport between Golgi and plasma membrane. It was the involvement of PLC which led us to study the role of the other member of this G protein family, the α subunits, in the regulation of membrane fission at the Golgi apparatus.

Results. Among constitutive active (QL) variants of different G protein α subunit sub-families, only GαqQL subunits were able to induce Golgi fragmentation, a phenotype that mainly reflects a membrane fission increase at this organelle. This phenotype was not observed with a GαqQL palmitoylation mutant, showing the need for a membrane-bounded subunit. Besides, GαqQL-dependent Golgi fission was blocked by specific PLC and PKC inhibitors, and in the presence of a PKD1-kinase dead variant. In addition, GαqQL was the only α subunit capable of inducing PKD1 phosphorylation. Finally, Vesicular Stomatitis Virus thermosensitive mutant glycoprotein (VSVG tsO45) transport assays have demonstrated that GαqQL acts directly on Golgi membranes to regulate trafficking between this organelle and plasma membrane.

Conclusions. All these results indicate Gαq subunits for the first time as a regulator of PKD-mediated intracellular trafficking between Golgi apparatus and plasma membrane, opening new perspectives in the understanding of internal trafficking regulation by external signals through G protein-coupled receptors.

Introduction

Since the first description of the secretory pathway by Jamieson and Palade in the 60s (Jamieson and Palade, 1966), and the model of protein sorting at the trans-Golgi network (TGN) proposed by Griffiths and Simons in the 80s (Griffiths and Simons, 1986), the protein trafficking field has been growing exponentially in the endeavour to explain how this complex process is regulated in eukaryotic cells, finding new components involved in this regulation.

One of the main discoveries in this area, achieved by Malhotra's laboratory in the 90s (Jamora et al., 1999), was the participation of protein kinase D1 (PKD1) in the regulation of vesicle fission at TGN, giving this multi-task protein a completely new role, exerted on this membranous organelle by direct kinase translocation. After years of research, a new trafficking regulatory pathway was finally delineated, suggesting that PKD1-dependent TGN membrane fission could be regulated by extracellular signals via G-protein-coupled receptor (GPCR) activation, and mediated by specific Gβγ subunits and novel protein kinases C (nPKCs) (Díaz Añel and Malhotra, 2005), phosphatidylinositol-dependent phospholipases C (PI-PLCs) (Díaz Añel, 2007), and diacylglycerol (DAG) (Baron and Malhotra, 2002).

Based on these results, especially those that have shown PLCβ involvement in PKD1-dependent trafficking regulation (Díaz Añel, 2007), we decided to investigate whether G protein α subunits had a

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Key words: Gαq, Golgi, PKD1, Trafficking.

Abbreviations used: BIM, bisindolylmaleimide; DAG, diacylglycerol; DEM, Dulbecco's modified Eagle medium; FBS, foetal bovine serum; GPCR, G-protein-coupled receptor; PKC, protein kinase C; PKD, protein kinase D; PLC, phospholipase C; PKD1, protein kinase D1; TGN, trans-Golgi network; WT, wild type

role in the formation of vesicles at the TGN, since some members of these signalling proteins are capable of activating PLCs in order to produce inositol-3-phosphate (IP_3) and DAG, where the latter has been shown to be absolutely necessary for the activation of novel PKCs and for PKD translocation to the TGN, both mechanisms that finally lead to membrane fission at this organelle.

Our current observations have shown that Gαq subunits have a similar role to specific Gβγ subunits, regulating PKD1-dependent vesicle fission at the TGN through PI-PLCs and PKCs, strongly suggesting that specific GPCR, and hence precise external ligands, may be involved in the regulation of intracellular vesicle trafficking, as proposed by several laboratories in recent years (Baldassarre et al., 2000; Sumara et al., 2009; Kong et al., 2010; Saini et al., 2010; Giannotta et al., 2012; Cancino et al., 2013).

Results

Constitutive active Gαq is the only α subunit capable of causing Golgi fragmentation

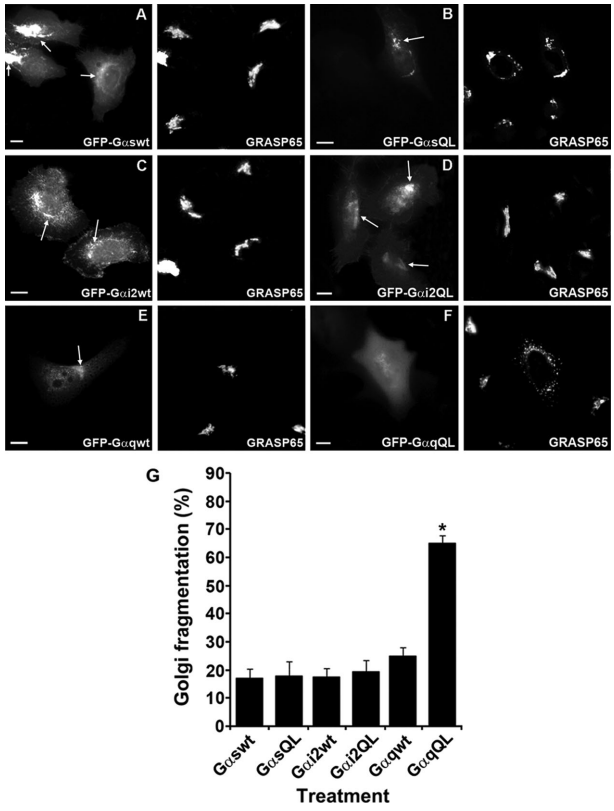
In order to study the effect of Gα subunits on Golgi structure, we transfected HeLa cells with constructs expressing different members of this family of proteins in both wild type (WT) and constitutive active versions, the latter being single amino-acid mutants known as QL (leucine for glutamine), which remains GTP-bound because of its deficient GTPase activity (Graziano and Gilman, 1989; Masters et al., 1989). These subunits were expressed as GFP fusion proteins to facilitate the identification of transfected cells by fluorescence microscopy.

We transfected both variants, WT and QL, separately, for G_{i2}, G_s, G_{OA}, G_{12} and G_q α subunits, observing that no WT proteins produced any change in Golgi structure (Figures 1A, 1C and 1E; G_{OA} and G_{12} not shown). QL mutants for the first four α subunits (G_{i2}, G_s, G_{OA} and G_{12}) showed the same result as their WT counterparts (Figures 1B and 1D; G_{OA} and G_{12} not shown). In contrast, overexpression of GαqQL induced a remarkable Golgi fragmentation (Figure 1F). Statistical comparison of Golgi fragmentation results between all Gα subunits are shown in Figure 1G.

For all the evaluated α subunits, co-localisation was observed between them and the Golgi apparatus (arrows in Figure 1A–1E). As it was observed for

Figure 1 | Constitutive active Gαq is the only α subunit capable of causing Golgi fragmentation

HeLa cells were transfected with GFP-GαsWT (**A**), GFP-GαsQL (**B**), GFP-Gαi2WT (**C**), GFP-Gαi2QL (**D**), GFP-GαqWT (**D**), GFP-GαqWT (**E**) or GFP-GαqQL (**F**) plasmids. 24 h post-transfection, cells were fixed and treated for immunofluorescence to detect GFP tag expression and Golgi apparatus (GRASP65, right of each picture). Arrows indicate co-localisation between Gα subunits and Golgi apparatus. (**G**) Percentage of Golgi fragmentation induced by expression of Gα subunits shown in (A) to (F). In all the experiments, two hundred GFP expressing cells were counted. Values in (G) are the means (±S.D., vertical bars) for four separate experiments. *$P < 0.05$ compared with GαqWT. Scale bars, 10 μm.

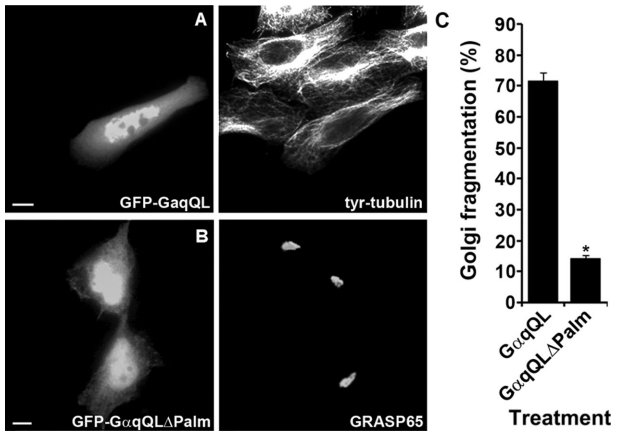


Gβ1γ2 (Díaz Añel and Malhotra, 2005), the wide degree of fragmentation achieved in the organelle by GαqQL overexpression, prevented the observation of co-localisation between this alpha subunit and the Golgi fragments (Figure 1F).

With the intention of comparing this result with those we observed for specific Gβγ subunits (Díaz Añel and Malhotra, 2005), we performed a number

Figure 2 | GαqQL activity does not affect microtubule cytoskeleton and it has to be attached to membranes in order to induce vesicle fission.

HeLa cells were transfected with GFP-GαqQL (**A**) and the Gαq constitutive active non-palmitoylable double mutant GFP-GαqQLΔPalm (**B**). In both experiments, 24 h post-transfection, cells were fixed and treated for immunofluorescence to detect GFP tag expression and microtubules (A: tyr-tubulin) or Golgi apparatus (B: GRASP65). (**C**) Percentage of Golgi fragmentation induced by GαqQL or GαqQLΔPalm expression. In all the experiments, two hundred GFP expressing cells were counted. Values in (C) are the means (±S.D., vertical bars) for four separate experiments. *$P < 0.05$ compared with GαqQL. Scale bars, 10 μm.



of controls to discard Golgi fragmentation being due to effects other than an induction of higher levels of vesicle fission in the TGN.

First of all, we tested whether GαqQL had any influence in tubulin polymerisation. Our results corroborated that, in the presence of the Gαq constitutive active mutant, the microtubule cytoskeleton remained intact (Figure 2A), as was previously observed with Gβ1γ2 overexpression (Díaz Añel and Malhotra, 2005), ruling out any effect of Gαq activity on microtubule scaffolding stability. We also ruled out Golgi fragmentation induced by cell death, since GαqQL expression had no effect on cell viability (data not shown).

Subsequently, we wanted to confirm that the effect of GαqQL was exerted specifically by its membrane localisation and not by protein overexpression. To do that, we created a new construct in which two amino terminal cysteine residues (numbers nine and ten) were removed from the original sequence of the QL mutant. This protein was identified as GFP-GαqQLΔPalm, since these two amino acids are required for Gαq palmitoylation, a post-translational modification essential for its binding to biological membranes. As shown in Figure 2B, GαqQLΔPalm did not co-localise with Golgi membranes, which remained intact when this mutant subunit was overexpressed, reinforcing the observation that the effect of these constitutive active mutants was due to the activation of a signalling pathway initiated by them in the membranes where they are bound, in a similar manner to that observed for Gβ1γ2 expressing cells in which the WT γ2 subunit was replaced by a geranylgeranylation mutant unable to attach to membranes (Díaz Añel and Malhotra, 2005).

All the above observations reinforce the participation of Gq alpha subunits in the regulation of membrane fission at the Golgi apparatus, and so we decided to proceed to investigate whether this signalling protein had a key role in PKD1-mediated trafficking control.

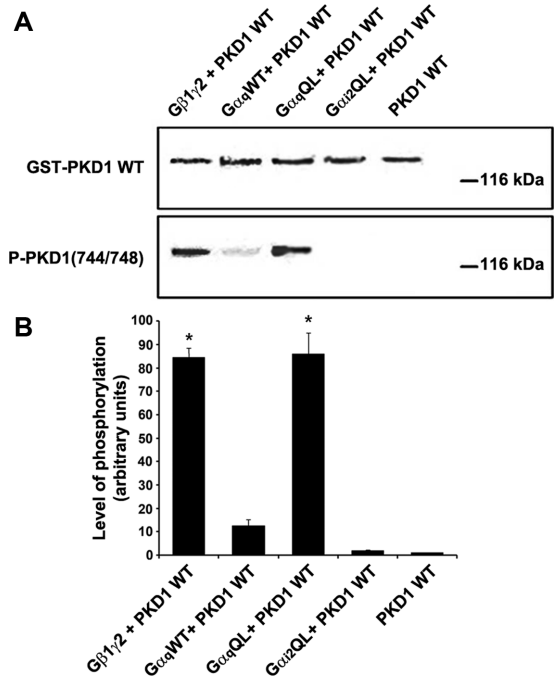
GαqQL acts on PKD1 to increase vesicle fission at the TGN

Previous studies of our laboratory have shown that Gβ1γ2-induced Golgi fragmentation was activated by PKD1 phosphorylation at its Serines 744 and 748, both located in the kinase activation loop (Díaz Añel and Malhotra, 2005; Díaz Añel, 2007). As this phosphorylation is first induced by interaction between Gβ1γ2 and PLCβ3, and taking into account that Gαq is also able to activate PLCβs, we decided to investigate whether PKD1 was also involved in Gαq-induced Golgi fragmentation.

To begin with, we transfected HeLa cells separately with GαqQL, GαqWT or $G\alpha_{i2}$QL, the latter as a negative control, together with GST-PKD1 WT. After 24 h of expression, we prepared RIPA extracts of the transfected cells and proceeded to run them for SDS-PAGE, in order to monitor PKD1 phosphorylation at its activation loop, with specific antibodies that detect PKD1 only when it is phosphorylated at Serines 744 and 748. Our results showed that GαqQL was able to induce phosphorylation of the PKD1 activation loop (Figure 3, third lane) at similar levels to those observed with Gβ1γ2 (Figure 3, first lane), whereas no phosphorylation was observed when $G\alpha_{i2}$QL was overexpressed (Figure 3, fourth

Figure 3 | GαqQL induces PKD1 activation loop phosphorylation at similar levels as Gβ1γ2

(**A**) HeLa cells were co-transfected with GST-PKD1 WT and FLAG-β1/HA-γ2 (first lane), GFP-GαqWT (second lane), GFP-GαqQL (third lane), GFP-Gαi2QL (fourth lane) or empty plasmid (fifth lane), and they were incubated for 24 h. Then, cell lysates were prepared and analysed by Western blot to monitor PKD1 activity (activation loop phosphorylation). (**B**) Densitometry of experiments described in (A), corresponding to GST-PKD1 WT phosphorylation at Serines 744–748. Values in (B) are the means (±S.D., vertical bars) for three separate experiments. *$P < 0.05$ compared with control (PKD1 WT)



lane). A basal PKD1 phosphorylation level was detected when GαqWT was expressed (Figure 3, second lane), an effect that might be due to the binding of endogenous GTP to this subunit, leading to a low but still noticeable amount of activated Gαq and, as a result, phosphorylated PKD1.

To confirm the role of PKD1 in Gαq-induced Golgi fragmentation, we decided to study the stability of this organelle structure in the presence of GαqQL when a PKD1 *dominant negative* form was co-expressed. HeLa cells were transfected with GαqQL and PKD1 WT or *kinase dead* PKD1 (PKD1 KD). In this KD construct, Lysine 618 has been replaced by Asparagine (K618N) at the adenosine triphosphate binding site, causing this protein to be inactive as a kinase (Liljedahl et al., 2001). As can be observed in Figure 4, while GαqQL co-expression with PKD1 WT showed no divergence with the result observed with GαqQL alone (Figure 4B), the presence of PKD1 KD completely blocked the effect of the constitutive active form of Gαq on Golgi fragmentation (Figure 4D), suggesting that this kinase mutant variant overexpression was capable of preventing the signalling pathway initiated by the activated form of Gαq, in addition to the fact that co-localisation was now observed between this constitutive active subunit, the Golgi-located PKD1 KD (Liljedahl et al., 2001) and the intact organelle (arrows in Figures 4C and 4D, respectively). Statistical analysis of Golgi fragmentation results from these experiments are shown in Figure 4E.

These results, comparable to those observed with Gβ1γ2, strongly indicate that Gαq is a main component of a signal transduction pathway that regulates vesicle fission at the TGN through PKD1 phosphorylation at its activation loop.

Gαq-induced Golgi fragmentation is PKC dependent

One of our previous works has demonstrated that PKD1 is activated by Gβ1γ2 and Gβ3γ2 complexes through PKCη (Díaz Añel and Malhotra, 2005), a kinase capable both of binding to the PKD1 PH (pleckstrin homology) domain and of directly associating to Golgi membranes.

Based on the action of GαqQL on vesicle fission at the TGN, and given the proven role of PKD1 in this process, we decided to verify if PKCs are involved in Gαq-mediated TGN fission regulation.

For this purpose, HeLa cells were treated with different PKC inhibitors after being transfected with GαqQL. The selected inhibitors were bisindolylmaleimide (BIM), tamoxifen and NPC15437, which block the activity of particular PKC isoforms, especially those belonging to conventional and novel subfamilies that are activated by DAG. Following the transfection of the cells with the constitutive active form of Gαq, they were directly incubated for 16 h with the corresponding inhibitors, and after that we proceeded to analyse the transfected cells by fluorescence microscopy in order to check the integrity of their Golgi apparatus.

Figure 4 | See Legend on the right

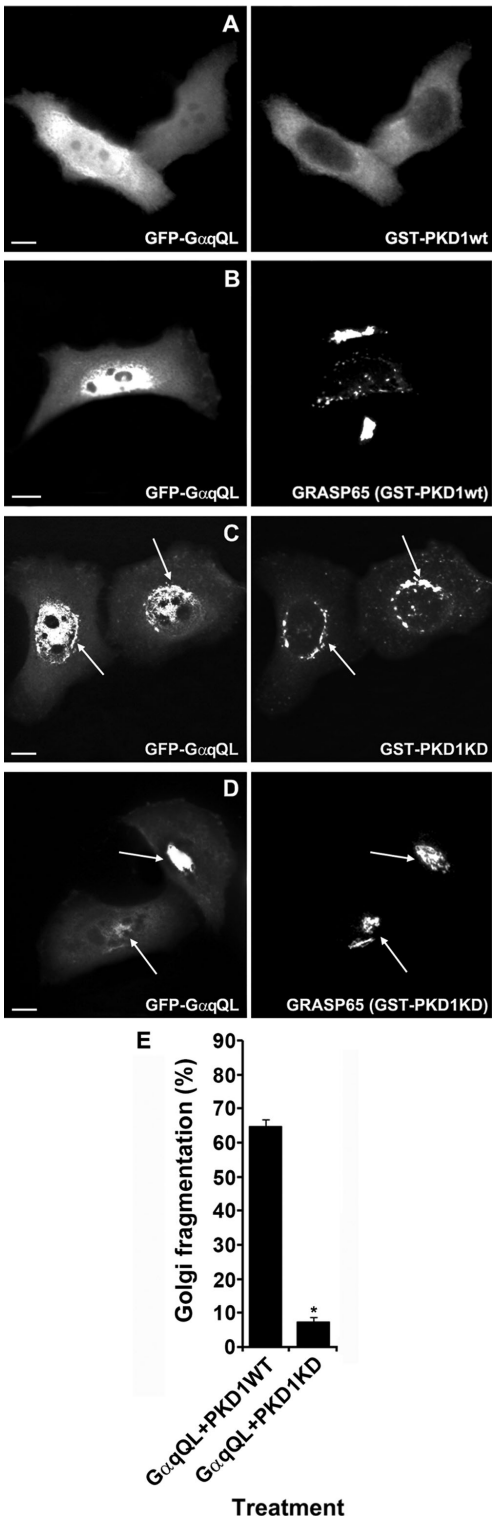


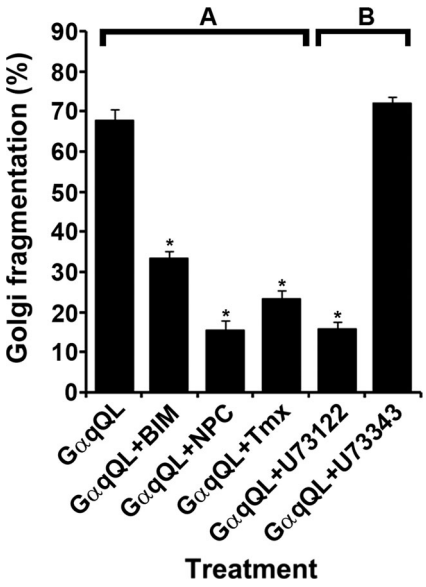
Figure 4 | GαqQL acts on PKD1 to increase vesicle fission at the TGN

HeLa cells were transfected with GFP-GαqQL and GST–PKD1 WT (**A and B**) or GST–PKD1 KD (**C and D**) plasmids. 24 h post-transfection, cells were fixed and treated for immunofluorescence to detect GFP tag expression (left image in **A** to **D**), PKD1 (GST, right image in **A** and **C**) and Golgi apparatus (GRASP65, right image in **B** and **D**). All GFP-GαqQL positive cells have also shown PKD1 WT or KD expression, therefore we assumed that all GαqQL positive cells who were monitored for Golgi structure status were expressing PKD1 WT or KD. Arrows indicate co-localisation between GαqQL subunit and PKD1 KD (**C**) or Golgi apparatus (**D**). (**E**) Percentage of Golgi fragmentation induced by GαqQL plus PKD1 WT or GαqQL plus PKD1 KD expression. In all the experiments, two hundred GFP expressing cells were counted. Values in (E) are the means (±S.D., vertical bars) for four separate experiments. $^*P < 0.05$ compared with GαqQL plus PKD1WT. Scale bars, 10 μm.

All three inhibitors were able to block Gαq QL-mediated Golgi fragmentation (Figure 5A), a result that was directly associated with the expected reduction of PKD1 phosphorylation at the activation loop (data not shown). Even though these inhibitors do not block single isoforms of PKC, based on those we have used it may be expected that the kinase involved in Gαq-mediated PKD1 activation would be among the conventional PKCα (inhibited by BIM), or novel PKCs δ (BIM and tamoxifen), ε (BIM) or η (tamoxifen and NPC15437), all of them described as PKD1 activators and with Golgi localisation (Aschrafi et al., 2003; Kajimoto et al., 2004; Li et al., 2004; Díaz Añel and Malhotra, 2005; Maissel et al., 2006; Berna et al., 2007; Poole et al., 2008; Garczarczyk et al., 2009), suggesting that any of these variants would be plausible candidates to participate in Gαq/PKD1-mediated Golgi fragmentation. Anyway, a work from Gonçalves laboratory described high specificity of tamoxifen and NPC15437 for inhibiting PKCη (Saraiva et al., 2003), which seems to strongly suggest that this PKC isoform is not only involved in G$\beta\gamma$-mediated Golgi membrane fission, but could also be part of a similar pathway initiated by Gαq subunits. The result with BIM could only be explained by the fact that there is currently no

Figure 5 | Gαq-induced Golgi fragmentation is PKC and PI-PLCs dependent

(**A**) HeLa cells were transfected with GFP-GαqQL plasmid. Subsequently, cells were incubated for 16 h with PKC inhibitors bisindolylmaleimide (BIM, second column), NPC15437 (NPC, third column), and tamoxifen (Tmx, fourth column) or their corresponding vehicles DMSO/water/ethanol respectively (DMSO is shown; first column). After the incubation, an immunofluorescence assay was performed to detect GFP tag expression (GαqQL) in order to measure Golgi fragmentation (GRASP65). (**B**) HeLa cells were transfected with GFP-GαqQL plasmid. Subsequently, cells were incubated for 16 h with PI-PLC inhibitor U73122 (left column), or its inactive analog U73343 (right column). After the incubation, an immunofluorescence assay was performed in order to detect GFP tag expression (GαqQL) and Golgi apparatus (GRASP65). In all the experiments, two hundred GFP expressing cells were counted. Values in (A) and (B) are the means (±S.D., vertical bars) for four separate experiments. *$P < 0.05$ compared with GαqQL.



clear data about an effect of this inhibitor on PKCη activity, which cannot be discarded. Even though further research will be necessary to identify the specific PKC isoform activated by Gαq. Our results with these PKC inhibitors would be very useful to narrow the quest for the involved kinase.

PI-PLCs are involved in Golgi vesicle fission induced by Gαq

The action of GαqQL on novel PKCs and PKD1, with the subsequent breakup of Golgi membranes, led us to search, as we did with Gβγ subunits, for the source of DAG, needed to both activate PKC and help PKD1 translocation to the TGN. As with our previous work, the logical candidate had to be a PI-PLC, since several isoforms of this PLC family are activated by Gαq, consequently leading to the production of second messengers DAG and IP_3 by hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$).

We decided to apply the same procedure that was performed with PKC activity-blocking drugs, which is the incubation of HeLa cells with a potent PI-PLC inhibitor, U73122, which has proven to be very accurate when used to investigate the role of these proteins in Gβγ-mediated trafficking regulation (Díaz Añel, 2007). GαqQL transfected cells were promptly incubated with U73122 and with its negative control U73343 for 16 h, and analysed by fluorescence microscopy with the intention of checking the stability of Golgi membranes.

This specific PI-PLC inhibitor was able to completely prevent GαqQL-mediated Golgi fragmentation, whereas in the presence of U73343, the inactive analog of U73122, the constitutive active form of Gαq showed a complete fragmentation effect on Golgi membranes (Figure 5B). These results seem to indicate that PI-PLCs are activated by Gαq and that they are responsible for the DAG production necessary to activate Golgi membrane fission through PKCs and PKD1 activation. Based on the activity of U73122 and the currently described PLCs that can be activated by this specific G protein alpha subunit, we were able to define PLCβ1, 3 or 4a (Kim et al., 1998; Rhee, 2001) as plausible candidate participants in the GαqQL-mediated trafficking regulation pathway.

As we stated in the preceding section, if we take into account that PKCη might be involved in both pathways, and that both Gβγ and Gαq are able to bind and activate PLCβ3, there is a possibility that both G protein subunits act on the same pathway through the activation of common GPCRs. As we have mentioned in the previous section, these results should be taken into account for future research aimed

to identify the specific PLCβ activated by Gαq in our pathway.

GαqQL regulates Golgi-to-plasma membrane transport

In order to analyse the effect of Gαq in TGN-to-plasma membrane transport, we decided to transfect HeLa cells with the temperature-sensitive mutant VSVGtsO45 and several G protein constructs.

When cells were transfected with Gαq WT, the Golgi-to-plasma membrane transport rate of VSVG reached normal values and the organelle remained intact (Figure 6A, second column), as shown previously in this manuscript (Figures 1E and 1G). The same results were observed when Gβ1γ2ΔC68S, the non-prenylated form of Gβ1γ2, was transfected (Figure 6A, third column). Previous work from our laboratory showed that this construct does not affect Golgi structure (Díaz Añel and Malhotra, 2005).

Even though GαqQL was able to fragment the Golgi during incubation at non-permissive temperature, nearly 50% of these cells showed VSVG reaching plasma membrane after incubation at 32°C, whereas the remaining viral protein was retained in TGN46 Golgi-positive vesicles (Figure 6A, sixth column). A similar result was obtained when Gβ1γ2 was expressed (Figure 6A, fourth column), as previously described by our laboratory (Díaz Añel and Malhotra, 2005; Díaz Añel, 2007). In both cases, co-transfection with kinase-dead PKD1 resulted in the blockage of VSVG Golgi-to-plasma membrane transport and the inhibition of Golgi fragmentation (Figure 6A, fifth and seventh columns), whereas both active subunits accumulated mainly in membranes of this organelle. These results demonstrate that neither of the constitutive active G protein subunits, GαqQL and Gβ1γ2, affected endoplasmic reticulum-to-Golgi transport, and that Golgi fragmentation is probably caused by the action of these subunits on PKD1 and hence on Golgi-to-plasma membrane transport, which continued working despite organelle breakup, albeit less efficiently.

Since Gβ1γ2ΔC68S showed no effect on transport, it was decided to co-transfect it with GαqQL in order to see if this constitutive active form could be blocked, based on previous reports that both subunits bind together after they are synthesised and transported via the anterograde trafficking pathway (Michaelson et al., 2002). In these conditions, Gβ1γ2ΔC68S was able to significantly block the action of GαqQL on Golgi, since the organelle remained intact (Figure 6B) and VSVG reached plasma membrane at normal rates (Figure 6A, last column). In these conditions, both subunits mainly localised at Golgi membranes (data not shown), which could be achieved only by GαqQL palmitoylation, verifying that this subunit can successfully attach to Golgi and exert its function directly on the organelle. Although Gβ1γ2ΔC68S is localised at Golgi membranes because of its binding to GαqQL, it cannot exert any action on transport, since for that it needs to be prenylated and unbound from Gα. These observations also demonstrate that, unlike WT Gαq bound to GTP, the constitutive form of Gαq is able to bind G$\beta\gamma$ subunits.

Discussion

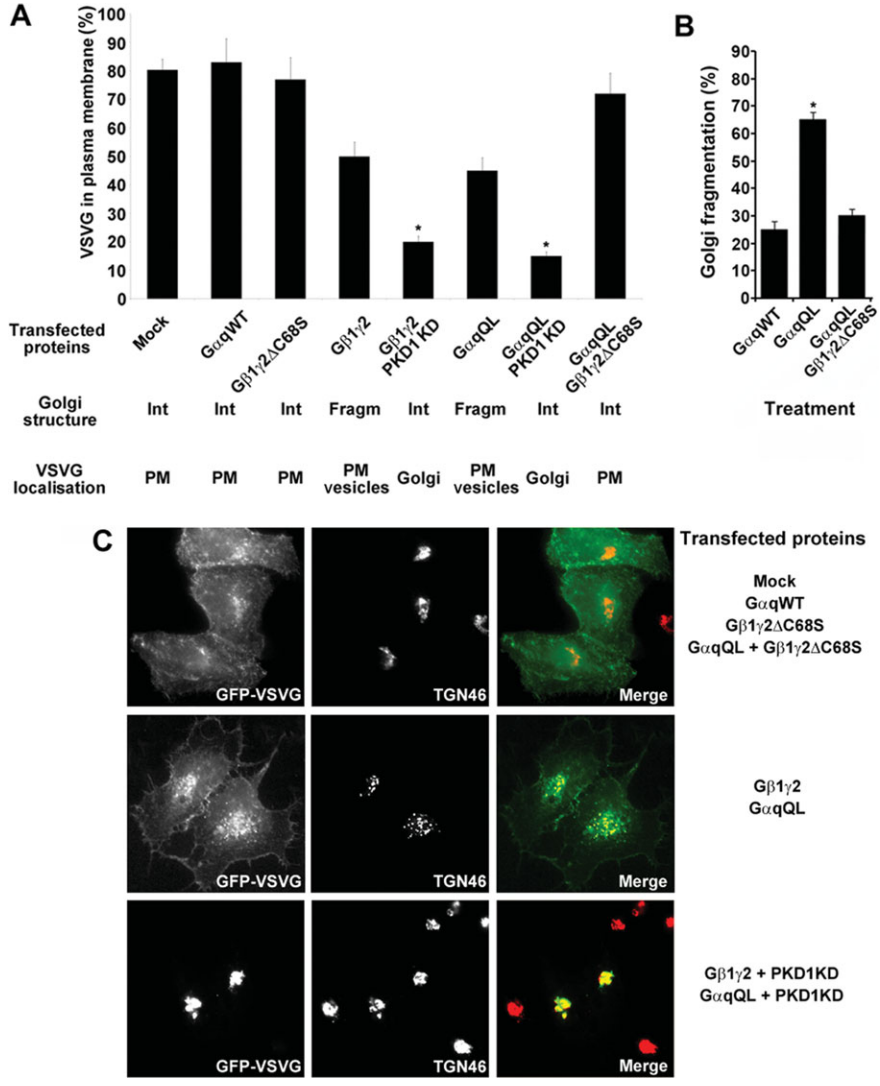
In recent years, it has been found that internal cellular trafficking can be regulated by the activation of plasma membrane or organelle-located receptors, particularly through one of its major effectors, heterotrimeric G proteins. The signalling cascades initiated by these particular proteins, which often exert their function on kinase systems and transcription factors, have been shown to play an essential role in regulating the fission of vesicles at the Golgi apparatus.

Our laboratory has shown that specific $\beta\gamma$ subunits of G proteins are able to induce vesicular fission in the Golgi through a signal transduction cascade involving PLCβ3, PKCη and PKD1. These results, together with many published recently showing that G$\beta\gamma$ subunits are able to translocate from plasma membrane to Golgi, strengthen our hypothesis that the rate of vesicular transport between these membrane organelles is regulated from outside of the cell through the activation of GPCR.

The participation of G protein-activated PI-PLCs in PKD1-mediated trafficking regulation led us to investigate whether the counterpart of G$\beta\gamma$, that is, Gα subunits, had any participation in these signal transduction pathways involved in the control of membrane fission. One of the obvious candidates to participate in these pathways would be Gαq, since this specific subunit activates members of the PLCβ family (Lee et al., 1993; Morris and Scarlata, 1997;

Figure 6 | GαqQL regulates Golgi-to-plasma membrane transport

(**A**) HeLa cells were co-transfected with different G protein subunits and VSVG-tsO45 constructs and incubated at non-permissive temperature (39.5°C) for 20 h. After adding cycloheximide, incubation continued for 30 more minutes at the same temperature. Then, they were incubated two more hours at permissive temperature (32°C), fixed and monitored by immunofluorescence. Measures of percentage of cells where VSVG had reached plasma membrane, final localisation of VSVG, and whether Golgi structure was intact or fragmented are shown in all the experiments. Int, intact Golgi; Fragm, fragmented Golgi; PM, plasma membrane localisation of VSVG; Vesicles, VSVG localised in Golgi-derived vesicles. In all experiments, two hundred G protein expressing cells (GFP for Gαq or HA for Gβγ) were counted. Values are means (±S.D., vertical bars) of four separate experiments. *$P < 0.05$ compared with its corresponding control (Gβ1γ2 or GαqQL) for VSVG transport blocking. Mock experiment corresponded to cells transfected only with VSVG. (**B**) HeLa cells were transfected with GFP-GαqWT, GFP-GαqQL or GFP-GαqQL plus Gβ1γ2ΔC68S plasmids. 24 h post-transfection, cells were fixed and treated for immunofluorescence to detect GFP tag expression and Golgi apparatus organisation. In all experiments, two hundred GFP expressing cells were counted. Values are the means (±S.D., vertical bars) for four separate experiments. *$P < 0.05$ compared with GαqWT. (**C**) Representative images of the three different phenotypes observed in transport experiments. Upper lane: intact Golgi and VSVG inserted at the plasma membrane; middle lane: fragmented Golgi and VSVG showing co-localisation with TGN vesicles and insertion in plasma membrane; lower lane: intact Golgi and VSVG retained at the TGN. To the right of each lane is indicated which plasmids were transfected in the experiments where those specific phenotypes were observed.



Golebiewska and Scarlata, 2010; Weinstein and Scarlata, 2011). To elucidate whether Gα subunits were involved in trafficking regulation, we selected representative subtypes of this vast protein family, and transfected HeLa cells with their constitutive active mutant (QL), which lacks GTPase activity and hence remains in a GTP-bound state.

As expected, of all the QL subunits tested, G_{i2}, G_s, G_{OA}, G_{12} and G_q, only the latter was able to generate Golgi fragmentation when overexpressed. This result was reinforced when a GαqQL palmitoylation mutant, unable to bind to membranes, had no effect on vesicle fission, indicating that this protein needs to be associated with biological membranes to initiate the pathway that leads to Golgi disassembling. These observations match the previous ones in which we showed that Gγ subunits have to be prenylated in order to activate, together with Gβ, the PLCβ3-PKCη-PKD1 pathway that regulates membrane fission at the TGN (Díaz Añel and Malhotra, 2005).

The role of Gαq was described in several works over the recent years, and it is clear that this protein acts directly on PI-PLCs to induce the production of the second messengers DAG and IP_3 (Golebiewska and Scarlata, 2010). This G protein subunit was also characterised as a PKD activator in different cellular processes (Yuan et al., 2000; Ozgen et al., 2008; Waldron et al., 2012), but its role in PKD-mediated protein trafficking was never acknowledged. Based on these points and taking into account our results with QL mutants, we investigated whether Gαq also initiated a pathway similar to the one described for G$\beta\gamma$.

First, blocking GαqQL-induced Golgi fragmentation by the inactive mutant of PKD1, plus the specific phosphorylation of this kinase at its activation loop by the presence of the G protein constitutive active mutant, demonstrated that these two proteins belong to a vesicle trafficking regulatory pathway, as was established for G$\beta\gamma$ subunits.

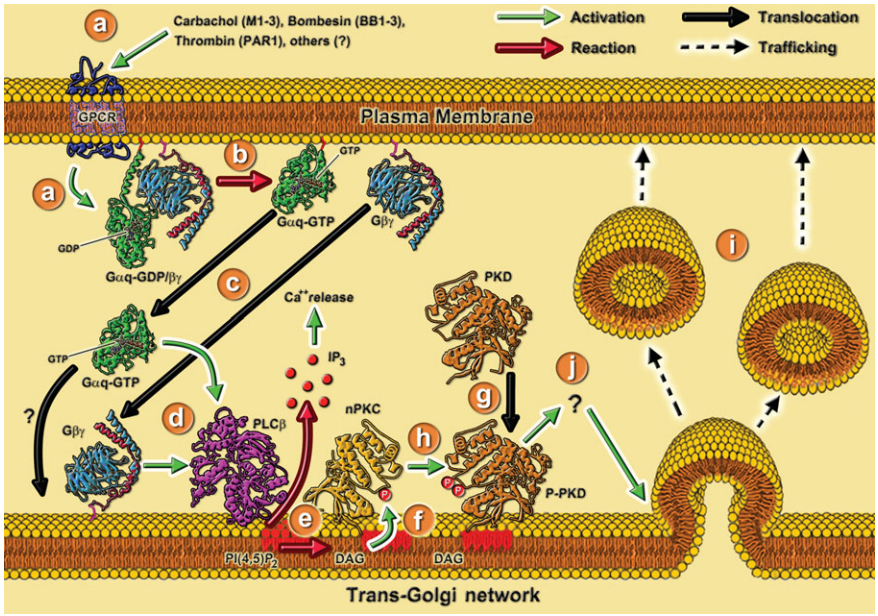
As in our previous works, and after confirmation of the role of Gαq and PKD1 in this pathway, we started to look for the PKD1 activator. We decided to use selected PKC inhibitors, because even though they have no specificity for single kinase isoforms, their combined results are a valuable tool to narrow the identification of the precise Gαq-mediated PKD1 activator. In addition, recent works have confirmed that two of the inhibitors employed, tamoxifen and NPC15437, have shown high specificity to block PKCη activity, which was not taken into account beforehand because this kinase isoform is abundant in epithelial cells, such as HeLa, and these inhibitors have apparently been tested in other cell types (Saraiva et al., 2003). This could be one of the reasons why we were able to detect blocking of GαqQL-mediated Golgi fragmentation by bisindolylmaleimide, despite the fact that this drug was not described as a PKCη inhibitor. Based on this and on our results with PKC inhibitors, we propose that PKCη is a strong candidate to participate in the pathway that regulates membrane fission at the Golgi apparatus through the activation of Gαq, although further research will be necessary to identify the specific PKC isoform activated by Gαq

In addition, as we have shown that PKCs and PKD1 are involved in Gαq-mediated vesicle fission at the TGN, we aimed at corroborating the role of the producers of the DAG necessary to make this pathway operate, which are PI-PLCs. A specific inhibitor for all the members of this phospholipase family has been able to prevent membrane fission at the Golgi apparatus induced by GαQL. Taking into consideration that Gαq was described as activating PLCβ1, β3 and β4a (Kim et al., 1998; Rhee, 2001), any one of these three proteins is a plausible candidate to initiate DAG synthesis by this G protein subunit.

Previous works have shown that PKD1 was able to regulate proteins that impact on DAG levels at the Golgi. One of these proteins, the lipid transfer protein CERT (ceramide transport; Fugmann et al., 2007), would be responsible for providing further DAG to the organelle, but after the recruitment of PKD1 to the TGN by a local DAG pool that can be generated via a different metabolic pathway, such as the one we are describing in this manuscript. The other protein, OSBP (oxysterol-binding protein, Nhek et al., 2010), have shown an important effect on lipid raft assembly and maintenance of lipid homeostasis when it was phosphorylated by PKD1, following the kinase recruitment to Golgi by DAG binding. Based on this, we believe that G proteins would directly activate the PLC beta pathway to increase the amount of DAG in the Golgi apparatus, so PKD1 can be recruited to this organelle in order to be activated, and thereafter to regulate vesicle fission, concentration of DAG and lipid homeostasis, through phosphorylation of substrates such as PI4KIII beta (see below),

Figure 7 | Model for TGN to plasma membrane transport regulation

We propose that an intracellular trafficking regulatory pathway is generated by still undefined ligands at the cell surface via GPCR (a). This signal produces the activation of a Gq protein (b), allowing its α subunit either to translocate to the TGN (c) or to be activated at the Golgi apparatus itself, where it is able to activate a PLCβ (1, 3 or 4a) by binding to its carboxyl-terminus domain (d). The activation of this phospholipase leads to the formation of DAG (e), which has two roles at the Golgi: to activate a novel PKC (most probably PKCη) (f), and to induce PKD1 to translocate to the TGN by direct binding of DAG to its C1a domain (g). After that, the novel PKC may be able to bind to the PKD1 PH domain, in order to phosphorylate its activation loop (h). Finally, PKD1 activation would induce cargo-filled vesicle fission at the TGN (i) by phosphorylation of downstream effectors, such as PI4KIIIβ and other still undetermined (j).



CERT and OSBP, respectively. Further research will be necessary to identify the specific PLC and PKC subunits involved in this pathway.

Finally, Gαq action has been shown to be exerted directly on the Golgi apparatus, because VSVG transport to plasma membrane can be restored by blocking GαqQL with a non-prenylated mutant of Gβ1γ2, while both subunits remain attached to the intact organelle. Kinase-dead PKD1 was also able to prevent the action of GαqQL on Golgi to plasma membrane transport, by blocking the last known step in the G-protein-regulated Golgi fission pathway.

In this manuscript, we have described a new role for Gαq, showing that these proteins are involved in the regulation of vesicle fission at the Golgi apparatus. Our results suggest that this Gα subunit is able to stimulate PKD1 phosphorylation at biological membranes through a clearly distinct signalling pathway, which includes a DAG generator that is a PI-PLCβ directly activated by Gαq, and a novel PKC, most probably PKCη. In this system, the DAG produced would have a double role, the activation of PKC and, as previously described (Griner and Kazanietz, 2007), the recruitment of PKD1 to Golgi membranes (Figure 7).

Even though new components for G protein-mediated trafficking regulation were described in recent years, there are still two points that need to be clarified in order to complete the signalling pathway. First, there are no newly described PKD1 substrates that can be characterised as responsible for membrane fission at the TGN. The closest candidate for this task is phosphatidylinositol-4 kinase III β (PI4KIIIβ), which is able to induce membrane fission after its activation by PKD1 (Hausser et al., 2005), but the mechanism by which this kinase produces new vesicles remains unknown. New research will be necessary to understand how Golgi membranes are bent

to generate vesicles, and which components are the PKD1 substrates that are involved in this process.

Secondly, one major objective will be to understand how this regulatory pathway is triggered at the plasma membrane according to the cell's growth and development needs. With the involvement of $G\beta\gamma$ this assignment became very difficult, since these subunits are usually very promiscuous because they interact with different GPCR and substrates, thus participating in several diverse pathways, such as $\beta1\gamma2$. But with our discovery of $G\alpha q$-mediated trafficking regulation, our quest narrows to a handful of GPCRs as candidates to initiate the signal from the cell surface or from internal organelles. A recent work has shown that activation of a particular GPCR at the plasma membrane, M3 muscarinic-receptor, induces PLCβ and PKD1-dependent Golgi fragmentation and insulin secretion (Saini et al., 2010). Although this work demonstrated mainly the role of $G\beta\gamma$ in secretion regulation, it is not odd to consider the participation of $G\alpha q$ in this mechanism, since M3 is associated with this type of $G\alpha$ subunit.

One of the controversial points about $G\alpha q$ participation in this regulatory pathway is its localisation. It is known that $G\beta\gamma$ can translocate from plasma membrane to Golgi once it has been activated (Akgoz et al., 2004; Akgoz et al., 2006; Chisari et al., 2007; Saini et al., 2007; Irannejad and Wedegaertner, 2010; Saini et al., 2010), but no comparable process was described for $G\alpha q$. However, a recent publication has shown that $G\alpha q$ cycles between the plasma membrane and Golgi apparatus, with the cycling depending on palmitoylation by the Golgi apparatus-resident proteins DHHC motif palmitoyl acyl-transferases 3 and 7 (DHHC3–7) (Tsutsumi et al., 2009), which would support the hypothesis that $G\alpha q$-associated GPCR can regulate intracellular trafficking from the cell surface. Anyway, in the case of $G\alpha q$ we cannot discard internal triggering, since a recent work demonstrated that this G protein subunit associates in the Golgi apparatus with KDEL receptors that behave as GPCR, and in this way they are able to regulate internal Golgi trafficking (Giannotta et al., 2012; Cancino et al., 2013). In these manuscripts, the authors demonstrated that $G\alpha q/11$ might have a role in intracellular transport via the KDEL receptor activation at the Golgi, but they did not elucidate by which mechanism exerts its effect on trafficking, hypothesising about its action on calcium release and a subsequent Golgi pool of Src family kinases activation. On the other hand, they have only evaluated $G\alpha q/11$ and $G\alpha s$, but did not analyse other G alpha subunits as it was done in our work, knowing that HeLa cells express a wide diversity of these subunits (Krumins and Gilman, 2006), and a number of these subunits (and not only $G\alpha q/11$ and $G\alpha s$) are localised at the Golgi, as it was shown by Michaelson et al., 2002, and as it was demonstrated for $G\alpha i$ by Lin et al., 2009.

All the results shown in this manuscript strengthen the role of heterotrimeric GTP binding proteins in vesicle fission regulation at the Golgi apparatus through PKD1 activation, and with the discovery of $G\alpha q$ participation in this regulatory pathway we are closing the gap that will allow us to understand how extra or intracellular signalling direct the trafficking rate inside the cell according to its requirements.

Materials and methods

Cell culture

HeLa cells (ATTC Nº CCL-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO; Cat. Nº 11995–065) supplemented with D-glucose and sodium pyruvate and 10% foetal bovine serum (FBS) and incubated at 37°C with 5% CO_2 atmosphere.

Cryopreserved cells were thawed by resuspension in DMEM at 37°C followed by incubation in a 100 mm dish. After reaching 90% confluence, they were divided in 35 mm dishes with 1 mm thick glass coverslips (Fisher Scientific, Cat. Nº 12–545–80.), for later use in plasmid transfection and protein expression experiments.

Dividing cells cultured in 100 mm dishes were washed in PBS (GIBCO, Cat. No. 70011–069) and treated for 5 min at 37°C with 2.5% Trypsin (GIBCO, Cat. Nº 15090–046). This protease treatment was inactivated by adding DMEM plus FBS. Cells were centrifuged at low velocity, the supernatant was discarded and the cell pellet was resuspended with fresh medium. Subsequently, cells were counted and plated in 35 mm dishes (4.2×10^5 cells, 35% confluence) or 24 multi-well at about 40% confluence in DMEM containing 10% FBS, and were allowed to grow to near confluence (24 h after plating). One hour before plasmid transfection, the media was changed to OPTI-MEM Reduced Serum Medium (GIBCO, Cat. Nº11058).

Constructs and cell transfection

The following green fluorescence protein-fusion tagged plasmids (pEGFP N2 expression vector; Clontech, Cat. Nº 6081–1, GenBank accession Nº U57608) for WT G protein alpha subunits were utilised: GFP-$G\alpha s$, GFP-$G\alpha i2$, GFP-$G\alpha q$, GFP-$G\alpha o$ and GFP-$G\alpha 12$. We also used their corresponding constitutive active mutants: GFP-$G\alpha$sQL, GFP-$G\alpha$i2QL, GFP-$G\alpha$qQL, GFP-$G\alpha$oQL and GFP-$G\alpha$12QL. In these constructs, the G protein

alpha subunit GTPase activity is inhibited by changing a leucine for a glutamine in the positions 227 (Gαs), 205 (Gαi2 and Gαo), 209 (Gαq) and 231 (Gα12), rendering these proteins in a constant GTP-bound (activated) state.

The GFP-GαqΔPalm mutant was created by deleting the double cysteine amino acids in the amino terminal portion of the G protein alpha q subunit. In physiological conditions, these cysteines are post-translationally palmitoylated in order to target the functional alpha subunit to biological membranes.

pCDNA3.1 plasmids expressing FLAG-β1 and HA (haemagglutinin)-γ2 were a gift from Silvio Gutkind [NIH (National Institutes of Health)]. PKD1 plasmids were constructed by Dr Yusuke Maeda (Research Institute for Microbial Diseases, University of Osaka). For trafficking assays, we made use of a VSVG thermosensitive mutant (tsO45) from our laboratory, where its GFP fussion tag was deleted.

For transfection experiments, we employed the Lipofectamine 2000^{TM} Transfection Reagent (Invitrogen, Cat. Nº 11668–019). A final concentration of 4 μg of DNA and 7 μl of Lipofectamine was used for a 35 mm plate or 1.6 μg/well of DNA and 1.5 μl of Lipofectamine for a 24 multi-well. Invitrogen recommended protocol was followed.

Twenty-four hours after transfection, cells were fixed with paraformaldehyde for immunofluorescence microscopy. In those cases where cell lysates for Western Blot were necessary, the procedure for transfection was performed directly on 35mm plates.

Immunofluorescence and Western blot

The following antibodies were used: rabbit polyclonal against GRASP65 protein (Golgi Re-Assembly Stacking Protein 1, 65 kDa) (Santa Cruz Biotechnology, Cat. Nº sc-30093), rabbit polyclonal anti-TGN46 (Sigma–Aldrich, Cat. Nº T7576), mouse monoclonal anti-tyrosine tubulin (Sigma–Aldrich, Cat. Nº T9028), Alexa Fluor 546 donkey anti-rabbit and Alexa Fluor goat anti-rabbit IgG secondary antibodies (Invitrogen, Cat. Nº A10040 and A21070, respectively), rabbit polyclonal anti-GST antibody (Santa Cruz Biotechnology, Cat. Nº sc-459), anti-phospho-PKD1 Ser744/748 (Cell Signaling, Cat. Nº 2054L), and a monoclonal anti-VSV-G Cy3-conjugated, clone P5D4 (Sigma–Aldrich, Cat. Nº C7706).

For immunofluorescence assays, transfected cells grown on coverslips were washed in PBS1X and fixed for 20 min in 4% paraformaldehyde–4% sucrose, followed by permeabilisation with Triton X-100 (0.02%) for 5 min, and blocking for one hour with BSA (5%) before incubation with the corresponding primary antibody. Appropriate primary antibodies were diluted 1:800 in BSA 1% and added to cells for 1 h incubation. After washing three times with PBS1X, cells were incubated for 1 h with the corresponding secondary antibodies in a 1:500 dilution, and mounted over slips with Fluor Save^{TM} Reagent (Calbiochem, Cat. Nº 345789–20ML), after washing twice with PBS1X. The entire procedure was performed at room temperature (25°C). Fixed cells were visualised in a Zeiss Axioplan Epifluorescence Microscope (Carl Zeiss Intl) with a 63X objective, numerical aperture 1.35, at room temperature. Pictures were taken with a 1344 × 1024 Orca ER CCD camera, and analysed with the Metamorph Software (Molecular Devices).

For Western blot assays, cells were transfected with GST-PKD1 WT and the corresponding Gα or G$\beta\gamma$ constructs. Twenty four hours after transfection, cells were washed twice with PBS1X and then they were lysed for 30 min at 4°C with 250 μl of RIPA1X buffer (NaCl 150 mM, Triton 1%, sodium deoxycholate, SDS 0.1%, Tris 50 mM, with protease inhibitors)/35 mm plate. Lysed cells were centrifuged at maximum speed, quantified with Lowry method, and diluted with the corresponding amount of Laemmli Buffer 1X to proceed with the SDS-PAGE. After the protein electrophoresis and transfer, the PVDF membrane was blocked 1 h at room temperature with non-fat dry milk 5% diluted in TBS-Tween 0.05% (500 mg non-fat dry milk, 10 ml TBS-Tween 0.05%). Then the membrane was incubated overnight at 4°C with the appropriate primary antibodies diluted in BSA according to manufacturer protocols. After five washes with TBS-Tween 0.05%, the membrane was incubated with secondary horseradish peroxidase conjugated-antibodies diluted in milk 1% for 1 h at room temperature. After another round of washes, the membrane was developed by chemiluminescence.

PKC and PI-PLC inhibitors

PKC inhibitors used for the experiments were: bisindolylmaleimide I (also known as GF109203X; Calbiochem, Nº 203290) prepared in DMSO (10 mg/ml) at a final concentration of 2 μM; NPC15437 (Sigma–Aldrich, Nº N161–5MG) prepared in water (10 mg/ml) at a final concentration of 10 μM; and tamoxifen (Calbiochem, Cat. Nº 579002–5MG) prepared in ethanol (5 mg/ml) with a final experimental concentration of 200 μM.

For the PI-PLC inhibition assay we used the specific inhibitor U73122 and its inactive analog U73343 (both from Calbiochem, Cat. Nº 662041 and 662035, respectively) prepared in chloroform at initial concentrations of 3 (U73122) and 200 mg/ml (U73343), with experimental concentrations of 1 mM for both compounds.

HeLa cells were cultivated and transfected in 24 multi-well with the corresponding GFP-Gαq plasmids. At the end of the procedure, the OPTI-MEM medium was changed for DMEM with the addition of the appropriate inhibitors in the final concentrations described in this section. After 24 h incubation, immunofluorescence assays were performed.

VSVG-tsO45 transport assay

HeLa cells were transfected with different G protein subunits and VSVG tsO45 constructs using Lipofectamine, and incubated at non-permissive temperature (39.5°C) for 20 h, according to Chi et al. (2008). Cycloheximide (Sigma–Aldrich, Cat. Nº C4859) was then added to the cell culture and incubation continued for thirty more minutes at the same temperature. Cells were incubated two more hours at permissive temperature (32°C) and, finally, they were fixed and checked by immunofluorescence. In all the experiments, three items were monitored: percentage of cells where VSVG had reached plasma membrane, final localisation of VSVG (only in those cases where plasma membrane localisation was observed in less than 75% of cells), and whether Golgi structure was intact or fragmented.

Author contribution

A.S.C. performed all the experiments. A.D.A. conceived the study and, together with A.S.C. and

M.L.M., participated in its design and coordination. A.D.A. drafted the initial manuscript. A.S.C., M.L.M. and A.D.A. edited the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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