The KDEL receptor couples to $G_{\alpha_{q/11}}$ to activate Src kinases and regulate transport through the Golgi

Monica Giannotta^{1,5,6}, Carmen Ruggiero^{1,5},
Mauro Grossi¹, Jorge Cancino²,
Mirco Capitani¹, Teodoro Pulvirenti^{1,7},
Grazia Maria Letizia Consoli³,
Corrada Geraci³, Francesca Fanelli⁴,
Alberto Luini^{2,*} and Michele Sallese^{1,*}

¹Unit of Genomic Approaches to Membrane Traffic, Department of Cellular and Translational Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro (CH), Italy, ²Department of Life Sciences, Institute of Protein Biochemistry, National Research Council (CNR) and Telethon Institute of Genetics and Medicine, Naples, Italy, ³Institute of Biomolecular Chemistry, National Research Council, Catania, Italy and ⁴Department of Chemistry, Dulbecco Telethon Institute (DTI), University of Modena e Reggio Emilia, Modena, Italy

Membrane trafficking involves large fluxes of cargo and membrane across separate compartments. These fluxes must be regulated by control systems to maintain homeostasis. While control systems for other key functions such as protein folding or the cell cycle are well known, the mechanisms that control secretory transport are poorly understood. We have previously described a signalling circuit operating at the Golgi complex that regulates intra-Golgi trafficking and is initiated by the KDEL receptor (KDEL-R), a protein previously known to mediate protein recycling from the Golgi to the endoplasmic reticulum (ER). Here, we investigated the KDEL-R signalling mechanism. We show that the KDEL-R is predicted to fold like a G-protein-coupled receptor (GPCR), and that it binds and activates the heterotrimeric signalling G-protein $G_{\alpha_{q/11}}$ which, in turn, regulates transport through the Golgi complex. These findings reveal an unexpected GPCR-like mode of action of the KDEL-R and shed light on a core molecular control mechanism of intra-Golgi traffic.

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*Corresponding authors. M Sallese, Unit of Genomic Approaches to Membrane Traffic, Department of Cellular and Translational Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro (CH) 66030, Italy. Tel.: +39 0872 570421; Fax: +39 0872 570412; E-mail: sallese@negrisud.it or A Luini, Department of Life Sciences, Institute of Protein Biochemistry, National Research Council (CNR) and Telethon, Institute of Genetics and Medicine, Via Pietro Castellino, Naples 80128, Italy. Tel.: +39 081 6132 535; Fax: +39 0871 560 9877; E-mail: luini@tigem.it

⁵These authors contributed equally to this work

⁶Present address: Unit of Vascular Biology, IFOM, The FIRC Institute of Molecular Oncology Foundation, Milan, Italy

⁷Present address: Cell Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

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Introduction

Complex machines, according to control theory (Sontag, 2005) must be governed by control systems for optimal functioning. This is likely to apply also to secretory membrane trafficking, which involves large and variable fluxes of proteins and lipids moving across anatomically separated membranous compartments. For the secretory pathway to maintain its size and composition and to respond properly to extracellular signals, such fluxes must be precisely controlled. The control of membrane fluxes across secretory stations might depend either on the levels and the self-assembly properties of the relevant molecular components, such as coat or SNARE proteins (Bonifacino and Glick, 2004; Heinrich and Rapoport, 2005), or on dedicated signalling circuits that monitor and govern transport rates to maintain homeostasis (Sallese *et al.*, 2006, 2009), or, more likely, on a combination of both mechanisms.

We have previously reported the existence of a signalling circuit that helps to maintain the dynamic equilibrium of trafficking through the Golgi complex (Pulvirenti *et al.*, 2008). In outline, we have proposed that a signalling cascade is initiated by transport itself, or, rather, by transport carriers that move out of the ER and carry chaperones leaving the ER to the Golgi, along with cargo proteins. At the Golgi, these chaperones bind the KDEL receptor (KDEL-R) through their C-terminal KDEL peptide. This causes the KDEL-R, a seven-transmembrane domain protein previously known to be involved in recycling the chaperones from the Golgi to the endoplasmic reticulum (ER), to initiate a signalling reaction that activates a Golgi pool of Src family kinases (SFKs). There are nine SFKs, several of which are partially functionally redundant (Thomas and Brugge, 1997). The activated Golgi SFKs initiate a phosphorylation cascade that promotes anterograde trafficking, thus balancing transport fluxes through the Golgi (Pulvirenti *et al.*, 2008). Moreover, we have shown that dynamin II is one of the (probably many) SFK phosphorylation substrates that control transport, as dynamin is phosphorylated by Src at tyrosine 231 and 597 and requires this phosphorylation to induce the fission of transport carriers that leave the Golgi (Weller *et al.*, 2010). The KDEL-R thus appears to be placed at the core of the Golgi transport control system. However, the mechanism by which it transduces traffic regulatory signals remains unknown.

Here, we report that (a) the KDEL-R interacts with, and (when bound to a KDEL-containing ligand) activates, the heterotrimeric G-protein $G_{\alpha_{q/11}}$; (b) $G_{\alpha_{q/11}}$ mediates the KDEL-R-induced activation of Golgi SFKs and of secretory trafficking; and (c) the KDEL-R possesses a (predicted) fold resembling that of G-protein-coupled receptors (GPCRs), a protein superfamily which comprises >1000 plasma membrane (PM) receptors and transduces a wealth of stimuli, including hormones, neuromodulators, immunomodulators, flavours and photons, by coupling to and activating the heterotrimeric G-proteins (Lefkowitz, 2007). These findings

reveal an unexpected GPCR-like signalling mechanism of action of the KDEL-R and begin to unravel the molecular control systems that operate in the secretory pathway.

Results

The KDEL-R coimmunoprecipitates with $G\alpha_{q/11}$

To uncover the KDEL-R transduction machinery, we first sought to identify signalling proteins interacting with the KDEL-R. We expressed and immunoprecipitated Myc-tagged KDEL-R (KDEL-R-Myc) from HeLa cells, and analysed the precipitate using both mass-spectrometry and western blotting with antibodies against transduction-related proteins. We found that the heterotrimeric G-protein signalling subunits $G\alpha_s$ and $G\alpha_{q/11}$ (Figure 1A) (but not $G\alpha_{i3/0}$) coprecipitate with the KDEL-R. We also showed that $G\alpha_{q/11}$ and $G\alpha_s$ localize at the Golgi in our HeLa cells (Supplementary Figure S1), in agreement with previous reports that several G-proteins localize at the Golgi and modulate traffic (Stow *et al*, 1991; Schwaninger *et al*, 1992; Pimplikar and Simons, 1993; Helms *et al*, 1998). These data thus suggest that the KDEL-R can interact with $G\alpha_{q/11}$ and $G\alpha_s$, a feature reminiscent of the GPCRs. Of note in this regard is that the KDEL-R is a seven-transmembrane domain receptor with an overall topology reminiscent of the GPCRs, although it lacks any detectable sequence homology of the GPCRs and it has never been proposed to belong to the GPCR superfamily (Graul and Sadee, 2001).

The predicted fold of the KDEL-R resembles that of GPCRs

We thus asked whether, in spite of the lack of sequence similarity the KDEL-R might have a GPCR-like topology, using a number of structural prediction techniques. Searching for templates through the available protein-fold recognition servers and meta-servers, we identified sensory rhodopsins II, a family of signalling proteins involved in bacterial phototaxis (Gordeliy *et al*, 2002), as the most meaningful template for modelling the seven-transmembrane regions of the KDEL-R. These rhodopsins, according to established databases, show the same topology/fold as bovine rhodopsin, a canonical GPCR (<http://scop.mrc-lmb.cam.ac.uk/scop/>; <http://www.cathdb.info/>). We thus built a KDEL-R model (Figure 1B and C) by integrating secondary structure predictions, comparative modelling, and *ab initio* loop modelling (see Materials and methods and Supplementary Figures S2 and S3A), and compared it with the structure of bovine rhodopsin (Supplementary Figure S3B and C). The two structures showed significant similarity in the transmembrane portions of their seven helices (with a C α -root mean square deviation relative to 147 superimposed pairs of 3.89 Å; Supplementary Figure S3B and C), suggesting that the KDEL-R and the GPCR superfamily share the same fold.

Traffic- and KDEL-R-induced activation of SFKs and of intra-Golgi traffic requires the active form of $G\alpha_{q/11}$

To examine whether the KDEL-R and the GPCRs also share the same mechanism of action, we first asked whether the KDEL-R requires $G\alpha_{q/11}$ and/or $G\alpha_s$ for signalling, that is, for activating the Golgi SFKs and for supporting intra-Golgi traffic (Pulvirenti *et al*, 2008). We knocked down $G\alpha_{q/11}$ or

$G\alpha_s$ by siRNA (Figure 1F), or inhibited the G_i and G_o isoforms by treating cells with pertussis toxin (PTX) (Stow *et al*, 1991), and then we activated the KDEL-R in these cells by generating a synchronized pulse of ER to Golgi traffic. To this end, we used a temperature-sensitive mutant of the vesicular stomatitis virus G glycoprotein (VSVG) as a synchronizable cargo, as described (Pulvirenti *et al*, 2008). While control cells as well as $G\alpha_s$ -depleted and PTX-treated cells exhibited similarly high levels of Golgi-SFKs activation, cells depleted of $G\alpha_{q/11}$ showed greatly reduced levels of SFKs activity (Figure 1D and E). To verify the specificity of the $G\alpha_{q/11}$ siRNA, we transfected $G\alpha_{q/11}$ -depleted cells with mouse $G\alpha_{11}$ (which differs in sequence from the human homologue and is not interfered by the human $G\alpha_{q/11}$ siRNA). The mouse $G\alpha_{11}$ rescued the traffic-dependent activation of SFKs at the Golgi complex (Supplementary Figure S4A–C). Finally, the role of $G\alpha_{q/11}$ was confirmed by monitoring SFKs activation by western blotting: in control cells, a traffic pulse activated the SFKs, whereas in $G\alpha_{q/11}$ -depleted cells no such activation occurred (Figure 2A). These data therefore show that the traffic- and KDEL-R-dependent activation of the Golgi SFKs requires $G\alpha_{q/11}$, but not $G\alpha_s$ or $G\alpha_i$.

Next, since we have previously shown that the activity of the Golgi SFKs is required for intra-Golgi trafficking (Pulvirenti *et al*, 2008), we examined the effects of $G\alpha_{q/11}$ and $G\alpha_s$ depletion on this traffic step. We first monitored transport using VSVG as a cargo. In cells depleted of $G\alpha_s$, traffic of VSVG through and out of the Golgi remained normal, while in $G\alpha_{q/11}$ -depleted cells it was inhibited (Figure 2B and C) (consistent with the SFKs activation data). Specifically, in $G\alpha_{q/11}$ -depleted cells VSVG reached the Golgi, but it then remained and accumulated there, and its arrival at the PM was strongly reduced (as assessed by antibodies against the extracellular portion of VSVG and by a surface biotinylation assay; Figure 2D and E; see Materials and methods). This block of intra-Golgi VSVG transport in $G\alpha_{q/11}$ -depleted cells was rescued by the transfection of mouse $G\alpha_{11}$ (Supplementary Figure S4D and E). Thus, the effects on traffic of $G\alpha_{q/11}$ depletion are quite similar to the effects of SFK inhibition (Pulvirenti *et al*, 2008), as expected.

We then examined the effects of $G\alpha_{q/11}$ depletion on traffic using two other different cargo types, namely, the endocytosis-defective mutant of the low-density lipoprotein receptor (LDLR-Y18A) and the soluble protein albumin–GFP chimera. We transfected these cargoes in $G\alpha_{q/11}$ -depleted HeLa cells. At steady state, both LDLR-Y18A and albumin were detectable in the Golgi complex in both control and $G\alpha_{q/11}$ -depleted cells, though at higher levels in the latter (Figure 3A and C). We then challenged the cells with cycloheximide to stop new cargo synthesis, and monitored the exit of the pre-existing cargo from the Golgi. In control cells, both LDLR-Y18A and albumin were transported normally, that is, LDLR-Y18A reached the PM and albumin was secreted (Figure 3A and C). In contrast, in $G\alpha_{q/11}$ -depleted cells both cargoes remained in the Golgi (Figure 3A–D). Thus, many cargo types require $G\alpha_{q/11}$ to move out of the Golgi.

To probe further the relationship between the KDEL-R and $G\alpha_{q/11}$, we used the $G\alpha$ subunit C-terminal peptides that have been developed to inhibit GPCR–G-protein signalling. These peptides mimicking critical portions of the interaction surface between specific G-proteins and GPCRs compete with this interaction (Gilchrist *et al*, 2002). We expressed the

Figure 1 The KDEL-R shares similarities with GPCRs and activates SFKs through $G\alpha_{q/11}$. (A) $G\alpha_{q/11}$ and $G\alpha_s$ coimmunoprecipitate with KDEL-R-myc from HeLa cells. The protein extracts (lysates) from wild-type HeLa (HeLa-control) and HeLa cells stably transfected with KDEL-R-myc (HeLa-myc) were immunoprecipitated with an anti-myc antibody. These lysates and the immunoprecipitates (anti-myc IPs) were analysed by western blotting using an anti-myc antibody (KDEL-R-myc) and antibodies against different $G\alpha$ classes, as indicated. (B) Cartoon representation of the structural model of the KDEL-R seen in a direction parallel to the membrane surface, with the intracellular side at the top. Helices 1, 2, 3, 4, 5, 6, and 7 are shown in blue, orange, green, pink, yellow, cyan, and violet, respectively. The N-terminal and C-terminal are red, intracellular loop 1 (IL1) and extracellular loop 2 (EL2) are lime, IL2 and EL3 are grey, and IL3 and EL3 are magenta. The side chains of the putative binding site amino acids (i.e., R5, D50, Y162, and N165; Scheel and Pelham, 1998) are shown as sticks; it can be seen that they are directed towards the core of the seven-helix bundle. (C) Cartoon representation of the structural model of the KDEL-R seen from the intracellular side, in a direction perpendicular to the membrane surface. (D) Depletion of $G\alpha_{q/11}$ inhibits traffic-pulse-dependent SFKs activation at the Golgi complex. HeLa cells were treated with non-targeting siRNAs (Ctrl), siRNAs against $G\alpha_{q/11}$ and $G\alpha_s$ ($G\alpha_{q/11}$, $G\alpha_s$ siRNA) for 72 h, or 400 ng/ml PTX for 16 h. After infection with VSV for 45 min, the cells were incubated at 40°C for 3 h (temperature block) and then shifted to 32°C for 30 min (block release). Control cells and siRNAs-treated cells were fixed and stained for active SFKs (p-SFKs, grey scale) and giantin (marker for Golgi area definition). Merged images following the temperature-block release are shown (p-SFKs/Giantin; red and green respectively). Scale bars, 10 μ m. (E) Quantification of data illustrated in (D). The p-SFKs IF intensities at the Golgi complex are expressed as arbitrary units (AU). Data are mean values ($\pm$ s.d.) from four independent experiments. *** $P < 0.001$ compared with 32°C control (ANOVA analysis). (F) Western blotting reveals decreases in $G\alpha_{q/11}$ and $G\alpha_s$ levels in siRNA-treated cells, compared with control cells. HeLa cells were treated with non-targeting siRNAs (Ctrl) and siRNAs against $G\alpha_{q/11}$ and $G\alpha_s$ (siRNA) for 72 h, and then homogenized. The cell lysates were analysed by immunoblotting for the different $G\alpha$ subunits, with actin as the loading control.

C-terminal undecapeptides of $G\alpha_{q/11}$ and $G\alpha_s$ ($G\alpha_{q/11}$ and $G\alpha_s$ ‘minigenes’) (Gilchrist *et al*, 2002), and examined their effects on the traffic- and KDEL-dependent Golgi-SFKs activation. The C-terminal peptide of $G\alpha_{q/11}$, but not that of $G\alpha_s$, inhibited the activation of SFKs at the Golgi (Figure 4A and B; Supplementary Figure S5A and B), confirming the role of $G\alpha_{q/11}$ and indicating that the traffic-dependent SFKs activation requires the same $G\alpha_{q/11}$ surface that participates in classical $G\alpha_{q/11}$ -GPCR interactions. The $G\alpha_{q/11}$ minigene, as expected, was also able to inhibit the transport of VSVG, which accumulated in the Golgi complex and did not reach the PM (Supplementary Figure S5C and D). These results are in agreement with our previous study (Pulvirenti *et al*, 2008), and indicate that this signalling is required for VSVG transport from the Golgi to the PM.

We also investigated whether $G\alpha_{q/11}$ is required in its ‘active’ (GTP-bound) conformation for KDEL-R-dependent signalling, as is the case for classical GPCR-G-protein systems. To this end, we first exploited the known interaction of $G\alpha_{q/11}$ with the RGS (regulator of G-protein signalling) domains of G-protein-receptor kinase 2 (GRK2) (Sallese *et al*, 2000). We and others (Sallese *et al*, 2000; Day *et al*, 2003; Sterne-Marr *et al*, 2003) have previously shown that the RGS domain of GRK2 (GRK2-RGS) selectively binds to the active (GTP-bound) form of $G\alpha_{q/11}$, but not to GDP-bound $G\alpha_{q/11}$. This interaction results in GRK2-mediated regulation of $G\alpha_{q/11}$ -coupled GPCRs by preventing activated $G\alpha_{q/11}$ from interacting with its effectors (Sallese *et al*, 2000). Thus, GRK2-RGS acts as an inhibitor of GTP-bound $G\alpha_{q/11}$ signalling. HeLa cells were thus transfected with GRK2-

Figure 2 Depletion of $G\alpha_{q/11}$ inhibits traffic-pulse-dependent SFKs activation and arrival of VSVG at the PM. **(A)** HeLa cells were treated with non-targeting siRNAs and siRNAs against $G\alpha_{q/11}$ for 72 h. After infection with VSV for 45 min, the cells were incubated at 40°C for 3 h (temperature block), shifted to 32°C for 30 min (temperature-block release) and homogenized. Control cells (Ctrl) and siRNA-treated cells ($G\alpha_{q/11}$ siRNA) were analysed by immunoblotting for total SFKs, the phosphorylated active forms of the SFKs (p-SFKs) and the $G\alpha_{q/11}$ silencing efficiency. **(B)** HeLa cells were treated with non-targeting siRNAs (Ctrl, upper panels), and with siRNAs against $G\alpha_s$ ($G\alpha_s$ siRNA, middle panels) and $G\alpha_{q/11}$ ($G\alpha_{q/11}$ siRNA, lower panels) for 72 h. After infection with VSV for 45 min, the cells were incubated at 40°C for 3 h (temperature block) and then shifted to 32°C for the indicated times (temperature-block release). Panels 30 min: control cells (Ctrl) and siRNA-treated cells ($G\alpha_s$ siRNA and $G\alpha_{q/11}$ siRNA, respectively) were fixed and stained for VSVG (green) and GM130 (marker for Golgi area definition, red). Merged images of red and green signals are shown (Total VSVG/GM130). Panels 100 min: immunostaining for total VSVG (grey scale), VSVG at the PM (revealed by an antibody against the extracellular domain of VSVG; External VSVG, grey scale) and GM130. Merged images of total VSVG (green), external VSVG (red) and GM130 (blue) signals are shown (VSVG/GM130). Scale bars, 10 μ m. **(C)** Quantification of data illustrated in **(B)**. VSVG IF intensities at the PM were calculated as the ratio of VSVG on the PM to the total VSVG, and expressed as AU. Data are mean values ($\pm$ s.d.) from three independent experiments. *** $P < 0.001$ compared with control (ANOVA analysis). **(D)** Western blotting reveals decrease in $G\alpha_{q/11}$ levels in siRNA-treated cells. HeLa cells were treated with non-targeting siRNAs (Ctrl) and $G\alpha_{q/11}$ siRNAs for 72 h. The cells lysates were analysed by immunoblotting for $G\alpha_{q/11}$, with actin as the loading control. **(E)** Biotinylation analysis of the VSVG at the PM. HeLa cells were treated as in **(D)** for 56 h, transfected with VSVG–GFP, incubated at 40°C for 16 h and then shifted to 32°C for 100 min. Surface proteins were labelled with biotin, VSVG–GFP was immunoprecipitated with an anti-GFP antibody and separated by gel electrophoresis. The biotinylated VSVG–GFP was detected by peroxidase-conjugated streptavidin. As a control, total immunoprecipitated VSVG–GFP was detected by an anti-GFP antibody. The data are a representative experiment, which was performed three times.

Figure 3 Depletion of $G\alpha_{q/11}$ inhibits the arrival of LDL receptor at the PM and the secretion of albumin. **(A)** HeLa cells were treated as in Figure 2A, the last 16 h were transfected with the endocytosis-defective GFP-tagged LDL receptor (LDLR-Y18A-GFP). Cells were then incubated for 2 h with cycloheximide (chx) at 37°C to inhibit protein synthesis, fixed and labelled with GM130 (marker for Golgi area definition, red). Single channel (LDLR-Y18A-GFP) in grey scale and coloured merged signals in the insets (LDLR-Y18A-GFP/GM130), are shown. White (Ctrl panel) and red ($G\alpha_{q/11}$ siRNA) arrowheads indicate the LDLR-Y18A at the PM. Scale bars, 10 μ m. **(B)** Quantification of data illustrated in **(A)**. Data are mean values ($\pm$ s.d.) from three independent experiments. *** $P < 0.001$ compared with control at 120 min (Student's *t*-test). **(C)** HeLa cells were treated as in **(A)**, the last 16 h were transfected with albumin-GFP. Cells were then incubated for 2 h with cycloheximide (chx) at 37°C to inhibit protein synthesis, fixed and labelled with GM130 (marker for Golgi area definition, red). Single channel (Albumin-GFP) in grey scale and coloured merged signals in the insets (Albumin-GFP/GM130) are shown. Scale bars, 10 μ m. **(D)** Quantification of data illustrated in **(C)**. Data are mean values ($\pm$ s.d.) from three independent experiments. *** $P < 0.001$ compared with control at 120 min (Student's *t*-test).

RGS-GFP, subjected to a traffic-pulse, and monitored for SFKs activation at the Golgi. In cells transfected with GRK2-RGS-GFP, the activation of the SFKs by traffic was inhibited (Figure 4C and D), confirming that active (GTP-bound) $G\alpha_{q/11}$ is indeed required for SFKs activation. As a control, VSVG was able to reach the Golgi complex, excluding pre-Golgi effects of GRK2-RGS-GFP.

Finally, we sought to assess whether GRK2-RGS-GFP can impair the signalling triggered by direct and acute stimulation of the KDEL-R with a KDEL peptide. A difficulty here was that the hydrophilic peptide KDEL does not permeate cell membranes. To overcome this problem, we synthesized the CFFKDEL peptide linked to C2-Bodipy (Bodipy-KDEL), a KDEL-R ligand that can permeate membranes and bind to the KDEL-R in living cells (Pap *et al*, 2001), and we treated the

cells with this peptide. Bodipy-KDEL was first validated by testing its ability to induce the redistribution of the KDEL-R from the Golgi to the ER (an event that follows the exposure of the KDEL-R to KDEL ligands and is associated with KDEL-R activation (Pulvirenti *et al*, 2008)). The KDEL-R started to leave the Golgi (i.e., to move to the ER) after 15 min of Bodipy-KDEL treatment and became very low in the Golgi after 35 min (Supplementary Figure S6A and B). Moreover, after 10 min of exposure to Bodipy-KDEL, an intense SFKs activity was triggered at the Golgi, which peaked at 15 min and remained stable for 35 min (Supplementary Figure S6C and D). Together, these results indicate that the Bodipy-KDEL indeed activates KDEL-R-dependent signalling. As a specificity control, a peptide that does not bind the KDEL-R, Bodipy-KDEA (Raykhel *et al*, 2007) did not affect the activation of the

Figure 4 $G\alpha_{q/11}$ is required for the traffic-pulse-dependent Golgi-SFKs activation. (A) Traffic-pulse-dependent Golgi-SFKs activation is prevented by the $G\alpha_{q/11}$ subunit C-terminal peptide. HeLa cells were cotransfected with GFP and the pcDNA3.1 vector as control, (pcDNA3.1/GFP) and with the C-terminal minigene vectors ($G\alpha_s$ CT/GFP, $G\alpha_{q/11}$ CT/GFP). The cells were then incubated at 40°C for 3 h (temperature block, resulting in procollagen IV accumulation in the ER), and then shifted to 32°C for 30 min (temperature-block release), and fixed and stained for active SFKs (p-SFKs, grey scale) and GM130 (marker for Golgi area definition). Merged images are shown (p-SFKs/GM130/GFP; red, blue and green respectively). Scale bars, 10 μ m. (B) Quantification of data illustrated in (A). The p-SFKs IF intensities at the Golgi complex are expressed as AU. Data are mean values ($\pm$ s.d.) from four independent experiments. *** $P < 0.001$ compared with 32°C control (ANOVA analysis). (C) Traffic-pulse-dependent Golgi-SFKs activation requires GTP-loaded $G\alpha_{q/11}$. HeLa cells were transfected with GFP (Ctrl) or GRK2-RGS-GFP-tagged constructs (GRK2-RGS-GFP). After 24 h, the cells were treated as in (A), fixed and stained for GM130 and active SFKs (p-SFKs, grey scale). Merged images are shown (GFP/p-SFKs/GM130; green, red and blue respectively). Scale bars, 10 μ m. (D) Quantification of the effects of GRK2-RGS-GFP transfection on p-SFKs in the Golgi area. The p-SFKs IF intensities at the Golgi complex are expressed as AU. Data are mean values ($\pm$ s.d.) from three independent experiments. *** $P < 0.001$ compared with 32°C control (ANOVA analysis). (E) The interaction of GRK2-RGS-GFP with $G\alpha_{q/11}$ increases in response to traffic-induced KDEL-R stimulation. HeLa cells were transfected with GRK2-RGS-GFP and after 48 h they were treated as in (A), and then homogenized either during the temperature block or 30 min after the temperature-block release (Traffic). The protein extracts were immunoprecipitated with an anti-GFP antibody and the immunoprecipitates (anti-GFP IP) were analysed by western blotting using an anti-GFP antibody (GRK2-RGS-GFP) and antibodies against $G\alpha_{q/11}$. As a positive control (Ctrl), the cells were treated with AlF_4^- and processed as above. NT stands for untreated.

SFKs or the levels of KDEL-R at the Golgi (Supplementary Figure S6). Finally, GRK2-RGS-GFP-transfected HeLa cells were treated for 15 min with 3 μ M of Bodipy-KDEL, fixed and SFKs activity evaluated by immunofluorescence (IF). In GRK2-RGS-GFP-transfected cells, the Bodipy-KDEL was unable to activate SFKs on the Golgi complex, confirming that KDEL-R stimulation activates the $G\alpha_{q/11}$ -mediated signalling at the Golgi (Supplementary Figure S7).

Traffic and KDEL-containing ligands promote the activation of $G\alpha_{q/11}$ at the Golgi complex

Along this line, we then examined whether traffic induces the activation of $G\alpha_{q/11}$ *in vivo* and whether this activation occurs at the Golgi complex. To this end, we first again exploited the ability of GRK2-RGS-GFP to selectively bind activated $G\alpha_{q/11}$ (Sallese *et al*, 2000; Sterne-Marr *et al*, 2003). The rationale here was that if $G\alpha_{q/11}$ is activated during a traffic pulse, its binding with GRK2-RGS-GFP during intense trafficking

should increase. HeLa cells were transiently transfected with GRK2-RGS-GFP and subjected to a VSVG traffic pulse; then, GRK2-RGS-GFP was immunoprecipitated to examine its interaction with $G\alpha_{q/11}$. Western blotting showed that $G\alpha_{q/11}$ coimmunoprecipitated with GRK2-RGS-GFP to a much higher extent during a traffic pulse than in traffic-blocked cells (40°C), indicating that $G\alpha_{q/11}$ is indeed activated by the pulse (Figure 4E). As a positive control, we treated the cells with AlF_4^- , which causes $G\alpha_{q/11}$ to enter an activated state ($G\alpha \cdot GDP \cdot AlF_4^-$, which is analogous to $G\alpha \cdot GTP$). AlF_4^- , like the traffic pulse, markedly enhanced the coprecipitation of $G\alpha_{q/11}$ with GRK2-RGS-GFP (Figure 4E; Berman *et al*, 1996). Second, to determine whether the traffic-induced activation of $G\alpha_{q/11}$ occurs at the Golgi, we examined whether GRK2-RGS-GFP would move to $G\alpha_{q/11}$ at the Golgi during a VSVG traffic pulse. In quiescent cells, GRK2-RGS-GFP resided in the cytoplasm and in the nucleus (Sterne-Marr *et al*, 2003). GRK2-RGS-GFP would be thus expected to shift

Figure 5 $G\alpha_{q/11}$ is activated at the Golgi complex by traffic and KDEL-R stimulation. (A) GRK2-RGS-GFP is recruited to the Golgi complex in response to traffic-induced KDEL-R stimulation. HeLa cells were transfected with GRK2-RGS-GFP and after 24 h the cells were incubated at 40°C for 3 h (temperature block, resulting in procollagen IV accumulation in the ER), then shifted to 32°C for the indicated times (temperature-block release) and fixed. The cells were stained for GM130 (marker for Golgi area definition). Merged images of GRK2-RGS-GFP (green) and GM130 (red) signals are shown (bottom panels). Scale bars, 10 μ m. (B) Quantification of recruitment of GRK2-RGS-GFP to the Golgi complex, as the ratio of its Golgi to cytosol IF. Data are mean values ($\pm$ s.d.) from three independent experiments. *** P < 0.001 compared with time 0 (ANOVA analysis). (C) GRK2-RGS-GFP is recruited to the Golgi complex in response to ssHRP^{KDEL}-induced KDEL-R stimulation. HeLa cells were cotransfected with GRK2-RGS-GFP and an empty vector (Ctrl, upper panels) or ssHRP^{KDEL} (lower panels), fixed and stained for the KDEL-R (marker for Golgi area definition) and HRP (right panel, grey scale). Merged images of GRK2-RGS-GFP (green) and KDEL-R (red) signals are shown (GRK2-RGS-GFP/KDEL-R, middle panels). Scale bars, 10 μ m. (D) Quantification of the recruitment of GRK2-RGS-GFP to the Golgi complex, as the ratio of Golgi to cytosol IF. Data are mean values ($\pm$ s.d.) from three independent experiments. *** P < 0.001 compared with empty vector (Student's t -test).

at least partially to the Golgi when the Golgi pool $G\alpha_{q/11}$ is activated by traffic. Indeed, within minutes of the beginning of the pulse, a fraction of the cytosolic GRK2-RGS-GFP shifted to the Golgi area (Figure 5A and B; Supplementary Figure S8A and B). The increase in Golgi GRK2-RGS-GFP was moderate (roughly 80% over control levels) and as such difficult to visualize, but it was reproducible and easy to quantify. This Golgi signal peaked at around 15 min, and decreased after 30 min. Thus, these data indicate that $G\alpha_{q/11}$ is activated at the Golgi by traffic (Figure 5A and B). Further along this line, we then used artificial KDEL-containing ligands to stimulate the KDEL-R more directly and monitor the Golgi redistribution of GRK2-RGS-GFP. First, HeLa cells were cotransfected with GRK2-RGS-GFP and with a soluble secreted variant of horseradish peroxidase bearing the KDEL motif at its C-terminus (ssHRP^{KDEL}). This construct has been previously shown to cycle between the ER and the Golgi, and to function as a long-acting artificial KDEL-R ligand (Pulvirenti *et al*, 2008). In ssHRP^{KDEL}-transfected cells, GRK2-RGS-GFP was clearly detected at the Golgi complex

over the cytosolic background (indicating $G\alpha_{q/11}$ activation there), whereas in control cells, the levels of GRK2-RGS-GFP at the Golgi were hardly detectable (Figure 5C and D).

Second, we sought to assess GRK2-RGS-GFP recruitment following direct and acute stimulation of the KDEL-R with a KDEL peptide. Thus, GRK2-RGS-GFP-transfected HeLa cells were treated for 30 min with 3 μ M of Bodipy-KDEL, fixed and evaluated by confocal microscopy to determine the localization of GRK2-RGS-GFP. Bodipy-KDEL recruited GRK2-RGS-GFP to the Golgi complex, confirming that KDEL-R stimulation activates $G\alpha_{q/11}$ at the Golgi (Figure 6A and B). However, it was difficult to visually appreciate the Golgi redistribution of this construct (see above). To overcome this problem, we monitored the live dynamics of GRK2-RGS-GFP following Bodipy-KDEL. The data showed a time-dependent accumulation of GRK2-RGS-GFP in the Golgi area, and, at the same time, a slight reduction of this construct in the cell periphery, including the PM area (indication that GPCR-linked $G\alpha_{q/11}$ at the PM are not activated by Bodipy-KDEL, Figure 6C–F).

Figure 6 $G\alpha_{q/11}$ is activated at the Golgi complex upon ligand binding to the KDEL-R. (A) HeLa cells were transfected with GRK2-RGS-GFP and after 24 h the cells were treated for 30 min with vehicle (Ctrl) or 3 μ M Bodipy-KDEL, fixed and labelled with GM130, (marker for Golgi area definition). Arrows indicate the Golgi area. Single channel (GRK2-RGS-GFP) in grey scale and coloured merged signals are shown (GRK2-RGS-GFP/GM130; green and red respectively). Scale bars, 10 μ m. (B) Quantification of data illustrated in (A). Data are mean values ($\pm$ s.e.) from three independent experiments. *** $P < 0.001$ compared with control (Student's t -test). (C) HeLa cells were cotransfected with GRK2-RGS-GFP and Sialyltransferase-RFP (ST-RFP) as Golgi marker, 24 h later the cells were treated with 3 μ M Bodipy-KDEL for the indicated time. The cells were recorded by live-cell imaging. The average GRK2-RGS-GFP fluorescence was measured on Golgi area (red circle) using ST-RFP as reference and cytosol (blue circle). The merged signals is shown. (D) The GRK2-RGS-GFP fluorescence of the experiment in (C) is shown in pseudocolour. Scale bars, 10 μ m. (E) Quantitative fluorescence of Golgi area and cytosol of the experiment shown in (C). (F) Quantitative average fluorescence of Golgi area, cytosol and PM (green ellipse) of cells treated as in (C). The graph shows the ratio between fluorescence at time 0 (F_0) and fluorescence at the different times (F_t). Data are mean values ($\pm$ s.e.) of 12 cells from two independent experiments.

Altogether, these findings indicate that the stimulation of the KDEL-R by traffic or by artificial KDEL-containing ligands in living cells leads to selective activation of $G\alpha_{q/11}$ and of SFKs at the Golgi complex, although we cannot formally exclude that the stimulation by traffic might be in part mediated, or potentiated, by traffic-related but KDEL-independent pathways.

Binding of KDEL ligand to KDEL-R induces $G\alpha_{q/11}$ activation

Finally, we wanted to study the $G\alpha_{q/11}$ activation by the KDEL-R in a simplified *in-vitro* system. To this end, we modified an *in-vitro* assay that is commonly employed to monitor the ability of GPCR ligands to induce [35 S]GTP γ S binding to GPCR-bound G-proteins (Harrison and Traynor, 2003) and used it to test whether a KDEL peptide would induce [35 S]GTP γ S loading onto $G\alpha_{q/11}$ in Golgi membranes.

A difficulty here was that isolated ER/Golgi membranes tend to seal into vesicles (microsomes), which are not easily permeated by hydrophilic molecules (Scheel and Pelham, 1998). This makes it impossible for two hydrophilic ligands such as a KDEL peptide and GTP γ S to simultaneously reach the KDEL-R binding site and $G\alpha_{q/11}$, respectively, at the two opposite sides of the vesicular membrane. To overcome this problem, we used the membrane-permeant Bodipy-KDEL (described above) as a KDEL-R ligand in the [35 S]GTP γ S-binding assay. Golgi-enriched membranes from rat liver (Taylor *et al*, 1997) were incubated with [35 S]GTP γ S in the presence or absence of Bodipy-KDEL, and then $G\alpha_{q/11}$ was immunoprecipitated from detergent-solubilised membranes to determine the $G\alpha_{q/11}$ -associated [35 S]GTP γ S. Bodipy-KDEL, but not Bodipy-KDEA, stimulated the [35 S]GTP γ S binding to $G\alpha_{q/11}$ in a dose-dependent manner (Figure 7A). To confirm the specificity of this reaction, we used both a characterized

Figure 7 Direct KDEL-R stimulation triggers the loading of [³⁵S]GTPγS into Gα_{q/11}. (A) Bodipy-KDEL induces the stimulation of [³⁵S]GTPγS binding to Gα_{q/11} in a dose-dependent manner. Golgi-enriched membranes from rat liver were incubated with [³⁵S]GTPγS in the presence of increasing concentrations of the membrane-permeant KDEL-R agonist Bodipy-KDEL (dark bars) or Bodipy-KDEA (white bars) as control. After 5 min at 30°C, the membranes were collected and solubilised, Gα_{q/11} was immunoprecipitated, and the Gα_{q/11}-associated radioactivity was measured. The data are mean values ± s.d. of triplicate determinations, performed three times. **P* < 0.05 and ****P* < 0.001, Bodipy-KDEL compared with Bodipy-KDEA at the given concentration. (B) An anti-KDEL-R antibody impairs [³⁵S]GTPγS binding to Gα_{q/11}. Golgi-enriched membranes from rat liver were incubated with [³⁵S]GTPγS in the presence of the membrane-permeant KDEL-R agonist Bodipy-KDEL (3 μM). Two hours after addition of non-related IgGs (non-related IgGs) or a KDEL-R antibody (anti-KDEL-R Ab) at 4°C, the membranes were treated as in (A), collected and solubilised, and Gα_{q/11} was immunoprecipitated and the Gα_{q/11}-associated radioactivity was measured. The data are mean values ± s.d. of triplicate determinations from a single representative experiment, which was performed three times. **P* < 0.05, Anti-KDEL-R Ab compared with non-related IgGs. (C) Golgi-enriched membranes from transfected HeLa cells. Total lysate (TL) and Golgi-enriched membranes (GEM) from mock-transfected (empty vector) or KDEL-R-mutant-transfected (KDEL-R-D193N) HeLa cells were solubilised and analysed by immunoblotting for TGN46, Gα_{q/11} and the KDEL-R. (D) The KDEL-R mutant D193N impairs [³⁵S]GTPγS binding to Gα_{q/11}. Golgi-enriched membranes from mock-transfected (empty vector) or KDEL-R-mutant-transfected (KDEL-R-D193N) HeLa cells were incubated with [³⁵S]GTPγS in the presence of Bodipy-KDEL and treated as in (A). The data are mean values ± s.d. of triplicate determinations, each performed three times. ***P* < 0.01, KDEL-R-D193N compared with empty vector. (E) HeLa cells stably expressing KDEL-R-myc were transfected with Gα_{q/11} or pcDNA3.1 vector as control (Ctrl). After 24 h, the KDEL-R-myc was immunoprecipitated with an anti-myc antibody. The immunoprecipitated proteins, associated to the agarose beads, were incubated with [³⁵S]GTPγS in the presence of the KDEL-R agonist Bodipy-KDEL (3 μM) (dark bars) or the control peptide Bodipy-KDEA (3 μM) (white bars). After 10 min at 30°C, the immunoprecipitate was washed, and the radioactivity associated was measured. The data are mean values ± s.d. of triplicate determinations from a single representative experiment, which was performed three times. ****P* < 0.001, Ctrl Bodipy-KDEL compared with Ctrl Bodipy-KDEA; ****P* < 0.001, Gα_{q/11} Bodipy-KDEL compared with Gα_{q/11} Bodipy-KDEA; ****P* < 0.001, Gα_{q/11} Bodipy-KDEL compared with Ctrl Bodipy-KDEL (ANOVA analysis). (F) HeLa cells stably expressing KDEL-R-myc were transfected with Gα_{q/11}. After 48 h, cells were treated with the membrane-permeant KDEL-R agonist Bodipy-KDEL (3 μM) or the control peptide Bodipy-KDEA (3 μM) at 37°C for 1 h. The cells were lysed (lysates), immunoprecipitated with anti-myc antibody (anti-myc IPs) and analysed by western blotting using an anti-myc antibody (KDEL-R-myc) and an anti-Gα_{q/11} antibody. Note that lysates and anti-myc immunoprecipitated bands display similar intensities because their autoradiography films have been exposed differently. The data are a representative experiment, which was performed twice.

neutralizing antibody against the cytosolic C-tail of the KDEL-R (Pulvirenti *et al*, 2008), and a dominant-negative (DN) KDEL-R mutant (KDEL-R-D193N, it binds the KDEL peptide but is unable to recycle to the ER (Townsend *et al*, 1993)) both of which are known to inhibit the KDEL-R in cell-based systems (Pulvirenti *et al*, 2008). The anti-KDEL-R antibody inhibited the Bodipy-KDEL-induced [³⁵S]GTPγS association

with Gα_{q/11} (while control antibody had no effect) (Figure 7B). For experiments with the DN mutant KDEL-R-D193N, we used Golgi-enriched membranes prepared from HeLa cells transfected with this mutant (see Materials and methods, (Balch *et al*, 1984) and Figure 7C). Here, although Gα_{q/11} was more abundant in KDEL-R-D193N-containing than in control membranes (Figure 7C), the KDEL-R-D193N

samples showed lower levels (by 40%) of Bodipy-KDEL-stimulated [35 S]GTP γ S binding to $G\alpha_{q/11}$ than control samples (Figure 7D).

Further along this line, we sought to test the ligand-dependent activity of the KDEL-R using a semi-purified system, that is, the KDEL-R $G\alpha_{q/11}$ coimmunoprecipitated complex. We reasoned that in such a complex $G\alpha_{q/11}$ should be in the inactive conformation and would presumably undergo guanine nucleotide exchange upon addition of a KDEL (but not of a KDEA) peptide to the immunoprecipitated receptor. We thus immunoprecipitated Myc-tagged KDEL-R (KDEL-R-Myc) from stably transfected HeLa cells and tested the incorporation of [35 S]GTP γ S in this precipitate. Bodipy-KDEL stimulated [35 S]GTP γ S incorporation in the KDEL-R-containing precipitate (Figure 7E), indicating that KDEL-R remains functional and coupled to GTPases even after precipitation. We then performed similar experiments in HeLa cells transfected with $G\alpha_{11}$ to selectively examine the GTP γ S loading of $G\alpha_{q/11}$. In $G\alpha_{11}$ -transfected cells, the amount of [35 S]GTP γ S incorporated into the immunoprecipitated complex upon exposure to Bodipy-KDEL increased five-fold as compared with untransfected cells, indicating a functional coupling between the KDEL-R and $G\alpha_{11}$. We conclude that the KDEL-R, like a classical GPCR, couples to $G\alpha_{q/11}$ and can act as an exchange factor for $G\alpha_{q/11}$ in an agonist-dependent manner.

Finally, since the physical coupling between many GPCRs and G-proteins decrease upon receptor activation (Okuma and Reisine, 1992), we examined whether also the KDEL-R- $G\alpha_{q/11}$ coupling depends on the activation state of the receptor. HeLa cells stably expressing KDEL-R-Myc were transiently transfected with $G\alpha_{11}$ and then treated with either Bodipy-KDEL or Bodipy-KDEA and immunoprecipitated with anti-Myc-conjugated agarose. As shown in Figure 7F, the amount of coimmunoprecipitated $G\alpha_{11}$ was slightly reduced in Bodipy-KDEL as compared with Bodipy-KDEA-treated cells, indicating that activated KDEL-R partially uncouples from the G-protein, again showing analogy of behaviour with that of the GPCRs.

Discussion

The central finding in this study is that the KDEL-R, a *cis*-Golgi protein involved in the sorting and retrieval of escaping chaperones back to the ER, operates also as a 'receptor', in a GPCR-like fashion, to sense incoming traffic and activate a pool of Golgi $G\alpha_{q/11}$ proteins, which then, as previously reported (Pulvirenti *et al*, 2008), initiate a signalling circuit that regulates further secretory traffic steps. A long list of converging *in-vitro* and *in-vivo* lines of evidence support this conclusion, as summarized in the following: (1) the fold of the KDEL-R resembles that of GPCRs, as predicted by a combination of fold recognition, secondary structure predictions, comparative modelling, and *ab initio* loop modelling; (2) the KDEL-R coimmunoprecipitates with $G\alpha_{q/11}$ and $G\alpha_s$; (3) $G\alpha_{q/11}$ and $G\alpha_s$ are present at the Golgi complex (4) ligand-activated KDEL-R induces the loading of GTP γ S onto $G\alpha_{q/11}$ in classical *in-vitro* GPCR activation assays; (5) the KDEL-R, once activated by traffic pulses or by specific ligands, promotes the activation of $G\alpha_{q/11}$ at the Golgi complex *in-vivo*; (6) the KDEL-R requires $G\alpha_{q/11}$ to exert its functional effects *in-vivo* (i.e., to activate SFKs at the Golgi

and to promote forward traffic); (7) these effects involve the active (GTP-bound) form of $G\alpha_{q/11}$; and (8) the KDEL-R activates $G\alpha_{q/11}$ via the same surface that participates in the activation of $G\alpha_{q/11}$ by 'classical' GPCRs.

Thus, at least from an operational point of view, the KDEL-R appears to be a GPCR. However, whether the KDEL-R is a bona fide GPCR, or whether it differs from classical GPCRs in as yet untested structural and mechanistic details, remains to be determined. In this regard, an interesting question for future studies relates to the temporal relationship between the activation of $G\alpha_{q/11}$ by the KDEL-R and the receptor's engagement by the COPI machinery for recycling to the ER (Majoul *et al*, 2001). Here, again, there might be an analogy with the classical GPCRs. For instance, for the PM receptors, the activation of the G-protein is an early event, followed by the engagement of the receptor by adaptor proteins (e.g., AP2, clathrin), which then promote the relocation of the receptor to the endosomal compartment. For the KDEL-R, we hypothesize an analogous cycle: the receptor might activate $G\alpha_{q/11}$ first and then be engaged by COPI, which would then drive the receptor relocation to the ER. A further related question concerns the mechanism by which the traffic-induced activation of $G\alpha_{q/11}$ activates the Golgi pool of SFKs. While elucidating this issue is beyond the scope of this work, a reasonable hypothesis can be formulated starting from the observation that traffic pulses induce Golgi-localized calcium increases (San Pietro *et al*, 2009; Micaroni *et al*, 2010). Since traffic, as noted above, activates $G\alpha_{q/11}$ at the Golgi, and $G\alpha_{q/11}$ can induce calcium release from intracellular stores (Hubbard and Hepler, 2006), it is logical to propose that the activation of $G\alpha_{q/11}$ might be responsible for the observed effect. Elevated calcium can then activate SFKs in multiple ways (Cattaneo *et al*, 2009), completing a sequence of events that, to summarize, starts from the KDEL-R and includes the activation of $G\alpha_{q/11}$ followed by a local calcium release. Clearly, further work is needed to verify this model and to uncover additional components that may link $G\alpha_{q/11}$ with SFKs at the Golgi during traffic.

In conclusion, the KDEL-R appears to possess functional and structural features that are reminiscent of those shown by the GPCR superfamily, and to regulate traffic through a GPCR-like mechanism. The control systems operating in membrane trafficking are still poorly understood, in contrast with the case of other major cell functions such as the cell cycle and ER protein folding (Alberts *et al*, 2002; Ron and Walter, 2007), whose regulatory mechanisms have been to large extent elucidated. The KDEL-R $G\alpha_{q/11}$ signalling machinery may open a new avenue to continue uncovering control components of the transport pathways.

Materials and methods

The detailed Materials and methods can be found in the Supplementary data.

Cell handling and transport protocols

HeLa cells (European Collection of Cell Cultures) were grown under standard conditions and transfected using the JetPEI reagent (Polyplus transfection, New York, USA) according to the manufacturer's instructions. VSV infection: cells were infected with VSV as described previously (Mironov *et al*, 2001). Transport-pulse protocols: PC-IV and VSVG transport pulses were performed as described previously (Mironov *et al*, 2001; Bonazzi *et al*, 2005). Intoxication with PTX: HeLa cells plated at 70–90% confluence

were treated with 400 ng/ml PTX (Calbiochem, San Diego, CA) for 16 h and then subjected to a traffic pulse. The steady-state transport of the endocytosis-defective LDL receptor (LDLR-Y18A) was performed as previously described (Cancino *et al*, 2007). The steady-state transport of albumin–GFP (prepared as described in Supplementary data) was performed as for the endocytosis-defective LDL receptor. siRNAs directed towards the $G_{\alpha s}$, $G_{\alpha q/11}$ (Krumins and Gilman, 2006) were purchased from Sigma-Aldrich and siCONTROL non-targeting siRNA no. 2 was from Dharmacon (CO, USA).

Preparation and uptake of membrane-permeant peptides

The conjugation of CFFKDEL and CFFKDEA peptides (Gen Script, USA) with Bodipy was performed as previously described (Pap *et al*, 2001). The cells were incubated with 1% FCS medium for 1 h, rinsed with PBS supplemented with 5 mM glucose, pH 7.4 and then incubated in PBS containing the Bodipy peptides (3 μ M).

Approaches to impair $G_{\alpha q/11}$ signalling

As first approach, HeLa cells were transfected with 50 nM of $G_{\alpha q/11}$ siRNAs using the Hyperfect (Qiagen) reagent according to the manufacturer's instructions. In all, 72 h later, the cells were infected with temperature-sensitive VSV, and subjected to VSVG transport protocol. For rescue experiments, $G_{\alpha q/11}$ interfered cells were transfected with mouse $G_{\alpha 11}$ (Origene Technologies) for 24 h, exposed to PC-IV traffic pulses, and processed for p-SFKs IF analysis. For rescue of VSVG transport, the cells were processed as described above. In all, 48 h after interference, the cells were transfected with G_{11} and temperature-sensitive VSVG–GFP and VSVG transport analysed by IF. As second approach, the $G_{\alpha q/11}$ subunit C-terminal peptide was used ($G_{\alpha q/11}$ CT) as previously described (Gilchrist *et al*, 2002). The third approach was based on the use of the GTP-bound $G_{\alpha q/11}$ signalling inhibitor GRK2-RGS. HeLa cells were transfected with GRK2-RGS–GFP for 24 h, subjected to PC IV transport synchronization protocol, fixed and stained for p-SFKs.

Protein analysis and immunoprecipitation

Cell lysates and western blotting were as previously described (Pulvirenti *et al*, 2008). $G_{\alpha q/11}$, SFKs (Santa Cruz Biotechnology), $G_{\alpha s}$ (Millipore), $G_{\alpha i3/0}$ (Upstate), p-SFKs (Invitrogen) and actin (Cell signalling) proteins, were detected using secondary horseradish peroxidase-conjugated (Calbiochem) and enhanced chemiluminescence substrate mixture (ECL Plus, Amersham). For immunoprecipitation, total cell lysates were prepared in lysis buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM β -mercaptoethanol, 1% Triton or 15 mM CHAPS (Sigma-Aldrich) and a cocktail of protease inhibitors, incubated with an agarose-coupled rabbit polyclonal c-myc antibody (50 μ l/mg) (Santa Cruz Inc.) or an agarose-coupled goat polyclonal GFP antibody (50 μ l/mg) (Vector Laboratories, Inc., CA). Bound proteins were eluted with 0.1 M ammonium hydroxide pH 11 or directly in $2 \times$ SDS-sample buffer and analysed by western blotting.

Biotinylation assay for surface VSVG detection

$G_{\alpha q/11}$ interfered cells were transfected with VSVG–GFP, incubated for 16 h at 40°C (to accumulate VSVG in the ER), then shifted to 32°C to initiate transport from the ER towards the PM. In all, 100 min after the release of the temperature-block, cells were washed with ice-cold PBS and incubated for 30 min with 0.55 mg/ml sulfo-NHS-biotin; the reaction was stopped with 100 mM glycine. The cells were lysed with 1% Triton X-100 in TBS-150 (50 mM Tris, 150 mM NaCl, pH 7.5) and incubated with and an agarose-conjugated GFP antibody (50 μ l/mg) (Vector Laboratories, Inc., CA). Immunoprecipitated beads were extensively washed with the lysis buffer, eluted in SDS-sample buffer and separated using the SDS–polyacrylamide gel electrophoresis. The VSVG–GFP on the cell surface was detected by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.), the total VSVG–GFP was detected with an anti-GFP antibody (Abcam, Cambridge, UK).

Microscopy

IF microscopy were as described previously (Pulvirenti *et al*, 2008). The Golgi G-proteins IF staining in Supplementary Figure S1 was obtained from streptolysin-O-permeabilized cells, as described

previously (Mironov *et al*, 1997). Quantification of IF in the Golgi area was defined by using Golgi-marker images (with GM130, BD Biosciences or giantin, Abcam). The total IF within each area of interest was acquired, and the IF intensity was calculated by integration of the IF signal within the region of interest divided by the area. VSVG on the PM was selectively stained using an anti-VSVG luminal domain antibody in non-permeabilized cells. Total VSVG was stained using the P5D4 anti-VSVG antibody after the cells had been permeabilized (Polishchuk *et al*, 2004). All experiments were carried out three times, and IF was quantified in at least 40 cells (from two wells) per point per experiment using the LSM510-3.2 software (Zeiss). The results are shown graphically on an arbitrary scale (AU).

Isolation of Golgi-enriched membranes

The isolation of rat liver Golgi membranes was performed as previously described (Taylor *et al*, 1997). Golgi fractions from HeLa cells were prepared following the method established by Balch *et al* (1984).

GTP γ S-binding assay

The [35 S]GTP γ S-binding assay follows the protocol previously described by Akam *et al* (2001). To detect [35 S]GTP γ S binding to $G_{\alpha q/11}$, Golgi enriched-membranes from rat liver or HeLa cells were washed and resuspended in assay buffer (100 mM NaCl, 10 mM $MgCl_2$, and 10 mM Hepes, pH 7.4) to a final protein concentration of 1 μ g/ μ l. In all, 100 and 30 μ g of Golgi enriched-membranes from rat liver and from HeLa cells, respectively, was used for each sample. These membranes were added to 50 μ l assay buffer containing 2 nM [35 S]GTP γ S (1000 Ci/mmol) (Perkin-Elmer, USA), 2 μ M GDP, without or with Bodipy-KDEL/KDEA (1–3 μ M), and/or non-related IgGs (6 μ g) or anti-KDEL-R Ab (2–6 μ g) and then incubated at 30°C for 5 min. Non-related IgGs and anti-KDEL-R Ab were preincubated for 2 h at 4°C. The $G_{\alpha q/11}$ was immunoprecipitated and the radioactivity was determined via scintillation counter.

To detect [35 S] GTP γ S incorporated into the immunoprecipitated KDEL-R complex, HeLa cells stably expressing KDEL-R–myc were transfected with $G_{\alpha 11}$ or pCDNA3.1 as control. After 24 h, the KDEL-R has been immunoprecipitated as described in protein analysis and immunoprecipitation. Agarose beads were extensively washed and used for the GTP γ S-binding assay. The reactions were terminated by the addition of ice-cold assay buffer, and the radioactivity determined via scintillation counter.

Computational modelling of the KDEL-R

The structural model of the KDEL-R was built by means of the comparative modelling software MODELLER (Sali and Blundell, 1993), by using the crystal structure of sensory rhodopsin II (i.e., PDB code: 1gue) as a template for the seven-helix bundle. Template selection privileged degree of completeness and quality of the structural model among the ones available in the PDB. Five of the six loops were modelled following an *ab initio* approach implemented in the same programme. To allow for *ab initio* loop modelling, the following portions characterized by lack of sequence similarity between template and target were eliminated from the template: 27–34 (i.e., first intracellular loop), 57–70 (first extracellular loop), 92–97 (second intracellular loop), 118–121 (second extracellular loop), 181–195 (third extracellular loop 3 and first six amino acids of helix 7), and 213–219 (C-terminal of helix 7). During comparative modelling, α -helical restraints were assigned to the KDEL-R sequences 20–25, 58–69, 115–122, 166–173, and 177–189 (i.e., C-term of H1, N-term of H3, N-term of H5, C-term of H6 and N-term of H7, respectively). Moreover, β -strand restraints were assigned to the 206–209 amino-acid stretch in the C-tail. Eleven different alignments and nine different distance restraints were probed, leading to the building of 2000 structural models. The alignment that led to the finally selected model is shown in Supplementary Figure S2A. Such model was finally subjected to refinement of the six loops (i.e., the 26–32, 52–57, 86–93, 141–145, and 173–177 amino-acid segments), by means of the LOOP routine within the MODELLER software (Fiser *et al*, 2000), leading to other 100 models. Amongst the first 15 models holding the lowest violation of stereochemical restraints, the one was selected that was characterized by an optimal combination of goodness of main chain torsion angles, consistency with secondary structure predictions, and high 3D Profile score. In this model, the highest

incongruence with secondary structure predictions concerns the second intracellular loop and helix 4 (Supplementary Figure S3A).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: MG, AL, and MS conceived and designed the study; M Grossi, CR, MG, MC, TP, and JC performed the experiments; GMLC and CG prepared the Bodipy-conjugated peptides; FF carried out the structure predictions; AL and MS prepared the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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