Regulation of secretory transport by protein kinase D–mediated phosphorylation of the ceramide transfer protein

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Protein kinase D (PKD) has been identified as a crucial regulator of secretory transport at the trans-Golgi network (TGN). Recruitment and activation of PKD at the TGN is mediated by the lipid diacylglycerol, a pool of which is generated by sphingomyelin synthase from ceramide and phosphatidylcholine. The nonvesicular transfer of ceramide from the endoplasmic reticulum to the Golgi complex is mediated by the lipid transfer protein CERT (ceramide transport). In this study, we identify CERT as a novel in vivo PKD substrate. Phosphorylation on serine 132 by PKD decreases the affinity of CERT toward its lipid target phosphatidylinositol 4-phosphate at Golgi membranes and reduces ceramide transfer activity, identifying PKD as a regulator of lipid homeostasis. We also show that CERT, in turn, is critical for PKD activation and PKD-dependent protein cargo transport to the plasma membrane. Thus, the interdependence of PKD and CERT is key to the maintenance of Golgi membrane integrity and secretory transport.

Introduction

PKD is a family of serine/threonine-specific protein kinases comprising three structurally related members: PKD1/PKC\(\mu\), PKD2, and PKD3/PKC\(\nu\). PKD contains two zinc–finger-like cysteine-rich motifs that bind DAG, a pleckstrin homology (PH), and a kinase domain. PKD localizes to the cytosol, nucleus, Golgi complex, and plasma membrane, where it regulates diverse cellular processes, including vesicle trafficking (Rykx et al., 2003; Wang, 2006). Thus far, only a few physiological PKD substrates are known (e.g., the neuronal protein Kidins220, the Ras effector RIN1, HDAC5, and PI4KIII\(\beta\); Iglesias et al., 2000; Wang et al., 2002; Vega et al., 2004; Hausser et al., 2005). At the TGN, PKD is critically involved in the fission of transport carriers en route to the cell surface (Liljedahl et al., 2001; Yeaman et al., 2004). PKD is recruited to the TGN by its cysteine-rich regions (Maeda et al., 2001; Baron and Malhotra, 2002; Hausser et al., 2002), where it is activated by PKC\(\eta\)-mediated phosphorylation (Diaz Anel and Malhotra, 2005). PKD-mediated phosphorylation of PI4KIII\(\beta\) stimulates its lipid kinase activity, resulting in enhanced phosphatidylinositol 4-phosphate (PI(4)P) production and cargo transport to the plasma membrane (Hausser et al., 2005).

In this study, we demonstrate that PKD also phosphorylates and regulates the activity of the Golgi-localized ceramide transfer protein (CERT; also known as Goodpasture antigen-binding protein), a cytosolic protein essential for the nonvesicular delivery of ceramide from its site of production at the ER to Golgi membranes, where conversion to sphingomyelin (SM) takes place (Hanada et al., 2003). Two CERT isoforms exist: the more abundantly expressed, alternatively spliced form missing a 26–amino acid serine-rich region and the full-length 624–amino acid protein, which is designated CERTL (Raya et al., 2000). Both CERT isoforms possess a steroidogenic acute regulatory lipid transfer (START) domain that is necessary and sufficient for ceramide binding and transport (Hanada et al., 2003). START domains are ~210 amino acids in length and form a hydrophobic tunnel that accommodates a monomeric lipid (Soccio and Breslow, 2003; Alpy and Tomasetto, 2005). They are found in 15 mammalian proteins, with CERT being most closely related to Pctp, which binds and shuttles phosphatidylcholine (PC) between membranes, and StarD10, a lipid transfer protein specific for PC and phosphatidylethanolamine (Soccio and Breslow, 2003; Olayioye et al., 2005; Wirtz, 2006). CERT proteins further contain an N-terminal PH domain with specificity for PI(4)P.

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Abbreviations used in this paper: KD, kinase dead; MLV, multilamellar vesicle; PC, phosphatidylcholine; PH, pleckstrin homology; PI4P, phosphatidylinositol 4-phosphate; SM, sphingomyelin; START, steroidogenic acute regulatory lipid transfer; TNP-PE, 2,4,6-trinitrophenyl-phosphatidylethanolamine; WT, wild type.

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that contributes to Golgi localization (Levine and Munro, 2002; Hanada et al., 2003) and an FFAT motif (two phenylalanines in an acidic tract) that targets the protein to the ER via interaction with the ER resident transmembrane proteins VAP-A and VAP-B (vesicle-associated membrane protein–associated protein; Loewen et al., 2003; Kawano et al., 2006). Nonvesicular lipid transfer is thought to occur at membrane contact sites, at which the ER comes into close apposition with other organelles (Levine and Loewen, 2006). CERT may thus shuttle a very short distance between ER and Golgi membranes or perhaps contact both compartments simultaneously. When overexpressed, the START domain of CERT is sufficient for ceramide transfer to the Golgi complex (Kawano et al., 2006). However, under physiological conditions, both Golgi and ER targeting motifs are essential for CERT function. In the CHO cell line LY-A, CERT was identified to contain a mutation within its PH domain (G67E), rendering the protein defective in PI(4)P binding, which resulted in reduced cellular SM levels (Hanada et al., 2003). The PI(4)P requirement for CERT function is further supported by a recent study showing that PI4KIIIβ activity is necessary for efficient ceramide trafficking to the Golgi (Toth et al., 2006). We now provide evidence that PKD phosphorylates CERT on serine 132 adjacent to the PH domain, whereby PI(4)P binding, Golgi targeting, and ceramide transfer activity are negatively regulated. Furthermore, by transferring ceramide that is required for DAG production to Golgi membranes, CERT stimulates PKD activity and ensures the maintenance of constitutive secretory transport.

Results and discussion

PKD is a key regulator at the Golgi complex, with PI4KIIIβ being the only local substrate identified thus far (Haussler et al., 2005). To test whether the Golgi complex–localized CERT protein may serve as a substrate for PKD, we made use of a phosphospecific substrate antibody, termed pMOTIF, that was raised against consensus motifs phosphorylated by PKD (Doppler et al., 2005). HEK293T cells were transfected with expression vectors encoding Flag-tagged CERT and CERTL. Immunoprecipitated CERT isoforms were analyzed by Western blotting with the pMOTIF antibody (Fig. 1 A). A pMOTIF signal corresponding to the molecular weight of CERT and, more weakly, to that of CERTL, was detected (Fig. 1 A). The weaker detection of the CERTL isoform by ~25% compared with CERT may be related to its known behavior to form aggregates, which may impact phosphosite accessibility to kinases (Raya et al., 2000). To investigate whether recognition of CERT by the pMOTIF antibody was dependent on PKD, we expressed CERT together with a kinase-dead (KD) dominant-negative PKD1 variant (PKD1-KD) in HEK293T cells. Coexpression of inactive PKD1 abolished CERT detection by the pMOTIF antibody, suggesting that the signal was indeed the result of PKD-mediated CERT phosphorylation (Fig. 1 B). To address the question of which PKD isoform was responsible for CERT phosphorylation, we used an RNAi approach to down-regulate PKD. Silencing of only one isoform did not influence the level of CERT phosphorylation as judged by immunoblotting with the pMOTIF antibody.

Figure 1. CERT is detected by a PKD substrate antibody. (A) HEK293T cells were transfected with expression plasmids encoding Flag-tagged CERT and CERTL. Cells were lysed, and CERT isoforms were immunoprecipitated with anti-Flag antibody. Immunoprecipitated proteins were subjected to SDS-PAGE followed by immunoblotting with PKD substrate antibody (pMOTIF; top) and, after stripping, with anti-Flag antibody (bottom). (B) HEK293T cells were transfected with Flag-CERT expression plasmid along with GFP-PKD1-KD or empty vector. CERT was analyzed by Western blotting as described in A. The expression of PKD1-KD was verified by immunoblotting with a PKD1-specific antibody (bottom). (C) HEK293T cells were either mock transfected or transfected with PKD1- and PKD2-specific siRNAs followed by transfection with Flag-CERT expression plasmid 48 h later. CERT phosphorylation was analyzed as described in A (top). Silencing of PKD1 and PKD2 was verified by immunoblotting of lysates with specific antibodies (bottom). The band marked with an asterisk is the result of non-specific binding. PKD1 is marked with an arrow. (D) HEK293T cells were transfected with Flag-CERT expression plasmid. Cells were left untreated (con) or were serum starved overnight followed by stimulation with either 10% serum for 2 and 6 h or 2.5 μg/ml 25-hydroxycholesterol for 1 h. CERT phosphorylation was analyzed as described in A. (E) COS7 cells expressing Flag-CERT and PKD1-GFP (top) or GFP-CERT (bottom) were fixed and stained with Flag and TGN46-specific antibodies (red), respectively. Bars, 10 μm.
The phosphorylation status of CERT was strongly reduced in serum-deprived cells and could be restored by the readdition of serum (Fig. 1 D), indicating that CERT phosphorylation is dependent on extracellular stimuli. It was recently reported that OSBP (oxysterol-binding protein) promotes CERT translocation to the Golgi complex in response to stimulation with its ligand, 25-hydroxycholesterol, thereby integrating sterol signaling and SM synthesis (Perry and Ridgway, 2006). In line with these studies, 25-hydroxycholesterol treatment was found to augment CERT phosphorylation (Fig. 1 D), possibly by bringing CERT to the Golgi in the vicinity of PKD. CERT has been demonstrated to colocalize with the cis/medial-Golgi marker GS28 (Hanada et al., 2003). Immunofluorescence analysis of GFP-tagged CERT expressed in COS7 cells showed that the protein localized to GS28-positive Golgi regions (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200612017/DC1). However, lipid transfer proteins are thought to act at membrane contact sites, which are formed between the ER and TGN (Levine and Loewen, 2006), where PKD is localized. Immuno-fluorescence staining of the membrane contact sites, which are formed between the ER and TGN (Levine and Loewen, 2006), where PKD is localized. Immuno-fluorescence staining of Flag-tagged CERT coexpressed with GFP-tagged PKD in COS7 cells revealed that the two proteins colocalize at the Golgi complex. Furthermore, staining of the TGN-specific marker protein TGN46 verified that CERT partially localizes to this compartment (Fig. 1 E).

To identify pMOTIF recognition sites in CERT, we searched for potential PKD consensus motifs characterized by a leucine, isoleucine, or valine residue in the −5 position and arginine in the −3 position relative to a serine or threonine. Two serines at positions 132 and 272 matching the PKD consensus motif (Fig. 2 A) were exchanged for alanines by site-directed mutagenesis. Mutants were expressed in HEK293T cells and tested for recognition by the pMOTIF antibody. Interestingly, mutation of serine 132 to alanine abrogated the detection of CERT with the pMOTIF antibody and caused an increase in electrophoretic mobility, which is indicative of the loss of phosphorylation, whereas the S272A mutation did not affect the pMOTIF signal (Fig. 2 B). On low percentage gels, the wild-type (WT) protein migrated as two distinct bands, indicating the presence of a phosphorylated and a nonphosphorylated CERT pool (unpublished data). To confirm that PKD was capable of directly phosphorylating serine 132, we performed in vitro kinase assays with purified PKD1 and recombinant CERT GST fusion proteins comprising the first 138 amino acids of the protein. WT CERT was efficiently phosphorylated by PKD1, whereas the CERT-S132A protein showed a strongly reduced incorporation of radioactivity in this assay (Fig. 2 C). Furthermore, in vitro PKD phosphorylation of WT but not CERT-S132A generated a recognition site for the pMOTIF antibody (Fig. 2 D). Collectively, these results prove that CERT is a genuine PKD substrate in vitro and in vivo and identify serine 132 as a specific PKD phosphorylation site in CERT that can be monitored with the pMOTIF antibody.

Serine 132 is in close proximity to the CERT PH domain (aa 23–117), making it possible that phosphorylation on this site affects PI(4)P binding by increasing the local negative charge. Therefore, we quantified PI(4)P binding of CERT-WT and -S132A by performing protein–lipid overlay assays. Cytosol from cells transiently expressing the CERT variants was incubated with membranes spotted with a concentration gradient of the different phosphoinositides, and bound CERT proteins were

![Figure 2](image-url)
detected via their GFP tag. As reported previously, the WT protein demonstrated weak binding to several phospholipid species but displayed strong interaction with PI(4)P (Levine and Munro, 2002; Hanada et al., 2003). CERT-S132A binding to PI(4)P was detectable at two- to fourfold lower concentrations as compared with that of the WT protein (Fig. 3 A). To corroborate these results, the association of CERT with multilamellar vesicles (MLVs) consisting of PC alone or PC plus 5% PI(4)P was measured. Although the addition of PI(4)P to PC vesicles increased the membrane binding of CERT-WT 1.5-fold, the binding of CERT-S132A was enhanced 1.9-fold, suggesting an increased affinity of the CERT-S132A mutant to PI(4)P (Fig. 3 B). To investigate whether this affected the association with Golgi membranes in intact cells, we performed fractionation studies with cells expressing CERT-WT and -S132A. To estimate the level of ER binding, we included a CERT mutant (G67E) defective in PI(4)P binding. Only a small proportion of the WT and G67E protein were recovered in the pellet fraction, suggesting that under the experimental conditions used, ER binding was negligible, and Golgi association of the WT protein was not maintained (Fig. 3 C). The CERT-S132A mutant protein was highly enriched in the pellet fraction, confirming that the enhanced affinity for PI(4)P stabilizes membrane association in vivo. Together, these data imply that CERT, once bound to the Golgi complex, is phosphorylated by PKD. This then decreases the affinity of CERT to PI(4)P and, thereby, regulates the interaction of CERT with the Golgi complex. Because PI(4)P is also present at the plasma membrane, additional factors must specify CERT targeting to the Golgi complex. One candidate is Arf1, which has been shown to interact with the structurally related proteins OSBP and FAPP1 (Levine and Munro, 2002). Whether CERT phosphorylation influences binding to such additional factors remains to be tested in the future.

The CERT protein has been shown to function as a lipid transfer protein (Hanada et al., 2003). Thus, we investigated whether CERT phosphorylation on serine 132 influenced its ability to bind and transfer ceramide between membranes. To this end, GFP-tagged versions of CERT-WT and -S132A were transiently expressed in HEK293T cells, and the cytosol fraction was analyzed for ceramide-specific lipid transfer activity using a fluorescence resonance energy transfer–based assay. In this assay, vesicles containing pyrene-labeled ceramide as a fluorescent donor and quenching amounts of 2,4,6-trinitrophenylphosphatidylethanolamine (TNP-PE) were used (Sommerharju, 2002; Olayioye et al., 2005). The lipid preparation used was total extract from porcine brain, which is likely to contain PI(4)P. Upon the addition of cytosol-containing CERT-WT, a steady increase in fluorescence was noted, which was not observed when control cytosol of vector-transfected cells was used (Fig. 3 D). Compared with the WT protein, CERT-S132A displayed a higher rate of lipid transfer, which was evident from a more rapid increase in pyrene fluorescence (Fig. 3 D). Similar results were obtained when 0.5% PI(4)P was added to donor liposomes (unpublished data). This suggests that CERT phosphorylation on serine 132 down-regulates ceramide transfer activity, most likely by decreasing association of the protein with membranes. Previous data have already shown that PKD regulates the level...
of PI(4)P at the Golgi complex by the phosphorylation-mediated activation of PI4KIIIβ (Hausser et al., 2005). Interestingly, PI4KIIIβ is critical for the transport of ceramide between the ER and the Golgi complex (Toth et al., 2006). Accordingly, together with the data presented in this study, a dual role for PKD in maintaining lipid homeostasis of Golgi membranes becomes apparent by controlling the on rate (via PI(4)P levels) and off rate (via direct phosphorylation) of CERT.

The transfer of ceramide from the ER to the TGN is essential for SM synthesis at this compartment (Hanada et al., 2003). Golgi-localized SM synthase 1 utilizes ceramide and PC to generate SM and DAG (Perry and Ridgway, 2005), the latter being a prerequisite for PKD recruitment and activation. Compounds that block DAG production at the TGN inhibit the binding of PKD to TGN membranes and interfere with secretory transport (Baron and Malhotra, 2002). Therefore, increased ceramide transfer from the ER to the TGN by the overexpression of CERT should result in an elevated local DAG pool and may consequently stimulate PKD activity and secretory transport. To test this hypothesis, we transiently expressed CERT-WT and -S132A in HEK293T cells and analyzed the autophosphorylation of endogenous PKD. Compared with the control, the expression of both CERT-WT and -S132A increased PKD activity, as revealed by analyses with a phosphospecific PKD antibody (Fig. 4 A). CERT has been reported to possess kinase activity (Raya et al., 2000), making it possible that it activates PKD by direct phosphorylation. However, kinase assays clearly demonstrated that PKD is not phosphorylated by CERT. Moreover, a kinase activity was associated with the CERT protein only under mild detergent conditions (Fig. S1). Thus, our results show that PKD activation is regulated by CERT proteins, most likely as a result of increased ceramide delivery and enforced SM/DAG synthesis. A similar function has recently been described for the lipid transfer protein Nir2 in the maintenance of DAG levels at the Golgi apparatus via regulation of the cytidine-5′-diphosphate–choline pathway (Litvak et al., 2005). RNAi-mediated knockdown of Nir2 decreased DAG and PKD levels at the Golgi complex and blocked secretory transport. Interestingly, this effect could be rescued by the addition of exogenous C₄-ceramide (Litvak et al., 2005), indicating a critical role for ceramide in DAG synthesis and PKD recruitment to the Golgi complex.

To address the question of whether CERT-mediated PKD activation indeed translated into enhanced secretory transport, we made use of a plasmid encoding HRP fused to a signal sequence (ss). The fusion protein ssHRP can be used as a reporter for constitutive protein secretion (Bard et al., 2006). In control cells, secretion of ssHRP could be detected within 1 h and increased over time (Fig. 4 B). Coexpression of PKD1-KD, which inhibits the secretory transport of cargo protein (Liljedahl et al., 2001; Hausser et al., 2005), almost entirely abrogated ssHRP secretion. This confirmed that HRP was secreted in a PKD-dependent manner in our assay. Coexpression of CERT-WT and -S132A strongly augmented the amount of secreted HRP (Fig. 4 B). Conversely, knockdown of CERT by RNAi in COS7 cells inhibited the secretion of HRP (Fig. 4 C and D), confirming the essential role for CERT in the constitutive exocytosis of cargo proteins. We could only detect a slight increase in secretion with the S132A mutant compared with the one observed with the WT protein. This is in accordance with the comparable activation of PKD by CERT-WT and -S132A (Fig. 4 A) but was unexpected in light of the substantially enhanced in vitro lipid transfer activity of the CERT mutant (Fig. 3 C). However, increased levels of ceramide may not necessarily translate into equivalent increases in DAG because DAG synthesis might be limited by the availability of PC and the activity of SM synthase.
The accumulation of ceramide is known to affect Golgi membrane stability and induces vesicle fission (Weigert et al., 1999; Fukunaga et al., 2000). Therefore, we investigated whether overexpression of the CERT-S132A mutant affected its localization and/or caused morphological changes of the Golgi apparatus. In addition to concentrating in GS28-positive regions of the Golgi complex, the CERT-S132A mutant displayed a dispersed punctate staining (Fig. 5 A). However, the distribution of GS28 itself and that of TGN46 was not affected by the expression of CERT-S132A, nor were these proteins present in the vesicular structures observed with the mutant CERT protein (Fig. 5 A). This rules out fragmentation of the Golgi apparatus as a consequence of CERT-S132A overexpression. Some of the vesicular structures were found to contain the cargo protein ssHRP, providing evidence that these structures represent Golgi-derived transport carriers (Fig. 5 A). It thus appears that the increased membrane affinity of CERT-S132A prevents its dissociation from budding vesicles. Interestingly, when co-expressed with CERT-S132A, the PH domain of OSBP also localized to these vesicles, indicating that these structures are PI(4)P positive (Fig. 5 B). The CERT-S132A mutant may therefore inhibit PI(4)P turnover, thus stabilizing the lipid on transport carriers. Of note, a CERT-S132E protein was indistinguishable from the alanine mutant in terms of cellular localization and, thus, could not be used to mimic the phosphorylated state (unpublished data).

Collectively, our data support the following working model: PKD is recruited to the TGN by a local DAG pool that can be generated via different metabolic pathways. PKD then activates PI4KIIIβ, increasing PI(4)P levels at the TGN. This, in turn, recruits the CERT protein to the Golgi complex, where it contributes to PKD activation and vesicular transport processes by providing ceramide as a precursor for further DAG production. The system is tightly regulated by a negative feedback loop: active PKD phosphorylates CERT at serine 132, thus decreasing the affinity of CERT toward its lipid target PI(4)P to ensure continuous rounds of lipid transfer from the ER to the Golgi compartment. In conclusion, we have...
identified CERT as a PKD substrate and provide evidence for a novel relationship between membrane lipid biogenesis and protein secretion.

Materials and methods

Immunofluorescence microscopy

Cells were fixed in 4% PFA for 10 min, washed, and incubated with PBS containing 0.1 M glycine for 15 min. Cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min and blocked with 2% goat serum in PBS containing 0.1% Tween 20 for 30 min. Cells were then incubated with primary antibody diluted in blocking buffer for 2 h followed by incubation with secondary antibodies diluted in blocking buffer for 1 h. Coverslips were mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) and analyzed on a confocal laser-scanning microscope (TCS SL; Leica) using 488- and 543-nm excitation and a 40/0.125 HCX PL APO objective lens. Images were processed with Photoshop (Adobe). All images shown are stacks of several confocal sections.

Protein extraction, immunoprecipitation, and Western blotting

Whole cell extracts were obtained by solubilizing cells in NP-40 extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate), plus Complete protease inhibitors [Roche]. Lysates were clarified by centrifugation at 16,000 g for 10 min. For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 2 h on ice. Immune complexes were collected with protein G-Sepharose beads [GE Healthcare] and washed three times with NP-40 extraction buffer. Whole cell extracts or immunoprecipitated proteins were subjected to SDS-PAGE, and proteins were blotted onto polyvinylidene difluoride membranes (Roth). After blocking with 0.5% blocking reagent [Roche] in PBS containing 0.1% Tween 20, filters were probed with specific antibodies. Proteins were visualized with HRP-coupled secondary antibody using the ECL system (Pierce Chemical Co.). Stripping of membranes was performed in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 60°C. Membranes were then reprobed with the indicated antibodies.

Recombinant protein purification and in vitro kinase assays

BL21 bacteria were transformed with pGEX6P-Flag-CERT-WT(1–138) and -S132A(1–138) vectors. Expression was induced with 0.5 mM IPTG for 4 h at 30°C. Bacteria were harvested and resuspended in PBS containing 50 μg/ml lysozyme, Complete protease inhibitors [Roche], 10 mM sodium fluoride, and 20 mM β-glycerophosphate. Triton X-100 was added to a final concentration of 1% before sonication. GST-CERT fusions were purified from clarified lysate with glutathione resin [GE Healthcare]. Recombinant proteins were incubated with purified PKD1 from insect cells in kinase buffer [50 mM Tris, pH 7.5, 10 mM MgCl₂, and 1 mM DTT] in the presence of either 2 μCi γ-[32P]ATP or 75 μM [32P]ATP at 25°C. Samples were resolved by SDS-PAGE, blotted onto membrane, analyzed on a phosphorimager (Storm 860; Molecular Dynamics), and detected with the indicated antibodies.

Cellular fractionation

Cells were harvested in hypotonic buffer (50 mM Tris, pH 7.4, containing Complete protease inhibitors, 1 mM PMSF, 5 mM β-glycerophosphate, and 5 mM sodium fluoride) and sheared by passage through a 25-G/16-mm needle. Nuclei were removed by centrifugation at 500 g cytosol from HEK293T cells transiently expressing GFP-tagged CERT and -S132A. Fluorescence intensities were normalized to the maximum intensity obtained after the addition of 0.5% Triton X-100 and the maximum GFP fluorescence to account for different protein expression levels.

Secretion assay

HEK293T cells were cotransfected with ssHRP-Flag plasmid together with empty vector, pEGFPN1-PKDI-KD, pDNA3-Flag-CERT-WT, and -S132A at a ratio of 1:6.5, respectively. For CERT RNAi, COS7 cells were transfected with ssHRP-Flag plasmid, harvested after 8 h, replated, and transfected with siRNAs. HEK293T and COS7 cells were washed with serum-free medium 24 and 48 h after transfection, respectively, and HRP secretion was quantitated by incubation of clarified cell supernatant with ECL reagent. Measurements were performed with a luminometer (Lucy2; Anthos) at 450 nm.

Online supplemental material

Fig. S1 shows that CERT does not phosphorylate PKD directly. Fig. S2 shows the colocalization of CERT-WT and G52B. Supplemental methods and materials provides information about the antibodies and reagents used, DNA constructs, and cell culture and transfection. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200612017/DC1.

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References


Supplemental materials

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Antibodies and reagents

Antibodies used in this study were as follows: rabbit anti-PKD substrate pAb (Cell Signaling), mouse anti-Flag and antitubulin mAbs (Sigma-Aldrich), mouse anti-GFP mAb (Roche), rabbit anti-PKD pAb (C-20; Santa Cruz Biotechnology, Inc.), rabbit anti-PKD2 pAb (Calbiochem), rabbit anti-CERT pAb (Bethyl Laboratories), sheep anti-TGN46 pAb (GeneTex), mouse antitransferrin receptor mAb (Zymed Laboratories), and mouse anti-GS28 mAb (BD Biosciences). The anti-pS910 PKD antibody monitoring PKD autophosphorylation has been described previously (Hausser et al., 2002). HRP-labeled secondary anti–mouse and anti–rabbit IgG antibodies were obtained from GE Healthcare, alkaline phosphatase–labeled secondary anti–mouse IgG antibody was purchased from Sigma-Aldrich, and AlexaFluor488- and -546–labeled secondary anti–mouse and anti–sheep IgG antibodies were purchased from Invitrogen. 25-hydroxycholesterol and egg PC were obtained from Sigma-Aldrich, porcine brain lipids (total extract) were obtained from Avanti Polar Lipids, Inc., and PI(4)P was obtained from Biomol.

DNA constructs

The CERT cDNA was amplified by PCR using pcDNA3-Flag-CERT (provided by J. Saus, Centro de Investigación Príncipe Felipe, Valencia, Spain) as a template with primers containing EcoRI restriction sites and cloned into the pEGFPC1 vector. Point mutants and truncated CERT variants were generated by QuikChange site-directed PCR mutagenesis (Stratagene). The Flag-CERT cDNA was subcloned into pGEX6P1 using EcoRI restriction sites. pEGFP-N1-PKD1-WT and -KD (K612W) have been described previously (Hausser et al., 2005). Primers used for site-directed PCR mutagenesis were as follows: CERT-S132A (5′-CGTGGAGCAGGCGCAATGGTGTCCCTGG-3′ and 5′-CCAGGGACACCATTGCGCATGCCAGCG-3′), CERT-S272A (5′-GGTTGATAGCAGGCGCTTGCGCAAGAGACTGG-3′ and 5′-CCAGTCTCTTCTGCCAGGCGTCCTCACGTTTAACC-3′), CERT-G67E (5′-CAGAGTATGGCTGCAGAGAATCCATCTGTCTTAGC-3′ and 5′-GCTAAGACAGATGGATTCTCTGCAGCATACTCTG-3′), and CERT truncations at amino acid 138 (5′-GGTGTCCCTGTTTGGTACGCAAGTGGCTACTC-3′ and 5′-GAGTAGCACCCTTGCTCAAGGACACCACCAGGGACACC-3′). All oligonucleotides were purchased from MWG Biotech. The ssHRP-Flag plasmid was provided by V. Malhotra (University of California, San Diego, La Jolla, CA); the plasmid encoding the GFP-tagged PH domain of OSBP was provided by T. Levine (University College London, London, UK).

Cell culture and transfection

HEK293T and COS7 cells were grown in RPMI + 10% FCS in a humified atmosphere containing 5% CO2. HEK293T cells were transfected using TransIT293 reagent (Mirus). For immunofluorescence, COS7 cells were grown on glass coverslips and transfected with Lipofectamine 2000 (Invitrogen). In the case of siRNA oligonucleotides, cells were transfected with Oligofectamine (Invitrogen). The siRNA oligonucleotides used were: PKD1 (5′-GUCGAGAAGAGUGCAAAATT-3′), PKD2 (5′-GCAAGACUGCAAGUUUAATT-3′), CERT (#1, 5′-CCACAUAGCUCUACUUAATT-3′; #2, 5′-GAACAGGAGGAGCAUUAATT-3′), and lacZ (5′-GCAGGAGCAGGAAUUACGCT-3′). All oligonucleotides were purchased from MWG Biotech.

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