

Role of the Second Cysteine-rich Domain and Pro275 in Protein Kinase D2 Interaction with ADP-Ribosylation Factor 1, *Trans*-Golgi Network Recruitment, and Protein Transport

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Submitted September 22, 2009; Revised December 7, 2009; Accepted January 12, 2010
Monitoring Editor: Benjamin S. Glick

Protein kinase D (PKD) isoenzymes regulate the formation of transport carriers from the *trans*-Golgi network (TGN) that are en route to the plasma membrane. The PKD C1a domain is required for the localization of PKDs at the TGN. However, the precise mechanism of how PKDs are recruited to the TGN is still elusive. Here, we report that ADP-ribosylation factor (ARF1), a small GTPase of the Ras superfamily and a key regulator of secretory traffic, specifically interacts with PKD isoenzymes. ARF1, but not ARF6, binds directly to the second cysteine-rich domain (C1b) of PKD2, and precisely to Pro275 within this domain. Pro275 in PKD2 is not only crucial for the PKD2-ARF1 interaction but also for PKD2 recruitment to and PKD2 function at the TGN, namely, protein transport to the plasma membrane. Our data suggest a novel model in which ARF1 recruits PKD2 to the TGN by binding to Pro275 in its C1b domain followed by anchoring of PKD2 in the TGN membranes via binding of its C1a domain to diacylglycerol. Both processes are critical for PKD2-mediated protein transport.

INTRODUCTION

The protein kinase D (PKD) family of serine/threonine kinases comprises PKD1/PKC μ , PKD2, and PKD3/PKC ν (Rykx *et al.*, 2003). PKDs are activated either directly via phorbol esters or indirectly by various mechanisms, including G protein-coupled receptors (Jamora *et al.*, 1999). These kinases are involved in various fundamental biological processes, including regulation of Golgi structure and function and consequently protein transport from the TGN to the plasma membrane (Rozenfurt *et al.*, 2005). At the TGN, PKDs are activated by G protein $\beta 1\gamma 2$ and $\beta 3\gamma 2$ subunits via the Golgi-associated nPKC η (Díaz Anel and Malhotra, 2005) and are required for the shedding of cargo containing vesicles from the TGN (Bard and Malhotra, 2006). This might be accomplished by PKD1-induced phosphorylation of phosphatidylinositol-4 kinase III β (PI4KIII β) (Hausser *et al.*, 2005) and ceramide transfer protein (CERT) (Fugmann *et al.*, 2007). Whereas there is substantial data on the function of PKDs at the TGN, the recruitment of these kinases to the TGN is less

well understood. PKDs consist of an N-terminal regulatory domain followed by two cysteine-rich zinc finger regions, termed C1a and a C1b domain; a pleckstrin homology domain; and the catalytic domain at the C terminus. Localization of PKD1 at the TGN requires its binding to diacylglycerol (DAG) via its first cysteine-rich domain (Maeda *et al.*, 2001; Baron and Malhotra, 2002). However, it is currently unclear how PKDs are targeted specifically to DAG at the Golgi. We were interested in the mechanism by which PKDs are recruited to the Golgi compartment. TGN recruitment of PKDs could be due to a particular local concentration of DAG or to the recruitment of PKDs by a specific Golgi resident protein. Because there is little evidence for the first assumption, we addressed the second option.

ADP-ribosylation factors (ARFs) are small GTPases that act as sensors of the lipid environment and transducers of information which results in changes in ARF activity and the consequent assembly of protein structures on the membranes. Based on their sequence homology, the human ARF proteins are classified into three classes: class I (ARF1 and ARF3), class II (ARF4 and ARF5), and class III (ARF6) (Moss and Vaughan, 1998). Both class I and II ARFs localize to the Golgi compartment and are thought to play overlapping and redundant roles (Gillingham and Munro, 2007). ARF6 is divergent from the other members localizing to the plasma membrane and the endocytic system. ARFs play a central role in the secretory pathway by regulating the membrane association and/or activation of many effector proteins such as coat proteins COPI (coatamer) (Donaldson *et al.*, 1992; Palmer *et al.*, 1993), clathrin adaptor proteins (Zhu *et al.*, 1998; Ooi *et al.*, 1998; Donaldson and Jackson, 2000; Boehm *et*

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-09-0814>) on January 20, 2010.

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Abbreviations used: ARF, ADP-ribosylation factor; DAG, diacylglycerol; PKD, protein kinase D; TGN, *trans*-Golgi network; VSV-G-GFP, green fluorescent protein-tagged ts045 mutant vesicular stomatitis virus-G protein.

al., 2001), Golgi-localizing, γ -adaptin ear homology domain, ARF-binding proteins (Dell'Angelica *et al.*, 2000), four-phosphate-adaptor proteins (Godi *et al.*, 2004), various lipid-modifying enzymes (Brown *et al.*, 1993; Honda *et al.*, 1999; Godi *et al.*, 1999), and other effectors (Boehm *et al.*, 2001). ARF6 is a major regulator of endocytosis, cytokinesis, and the organization of the actin cytoskeleton (Donaldson, 2003). Because class I and II ARFs localize to the Golgi and ARF1 plays a key role in the recruitment of various complexes of cytosolic proteins, including PKD substrates such as PI4KIII β to the Golgi network (Godi *et al.*, 1999; Hausser *et al.*, 2005; D'Souza-Schorey and Chavrier, 2006), we examined a potential role of ARFs in recruiting PKDs to the plasma membrane at the example of PKD2.

In the present study, we show that ARF1 specifically and directly interacts with PKD2. Furthermore, ARF1 and PKDs colocalize at the TGN in immunocytochemistry. The interaction of PKD2 with ARF1 is mediated via its C1b domain and regulated by ARF1 activity. We identify Pro275 within the C1b domain of PKD2 as the crucial amino acid for ARF1 binding, recruitment of PKD2 to the TGN and PKD2 function at the Golgi, i.e., the regulation of protein transport from the TGN to the plasma membrane. Our results provide a novel model in which the localization of PKD2 to the TGN is accomplished by both, the C1a and C1b domain of the kinase. The C1b domain of PKD2 interacts with ARF1 that recruits PKD2 to the TGN where the kinase is anchored by binding to local DAG via its C1a domain.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa and HEK293-T-cells were grown in DMEM with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 10% CO₂ at 37°C. Exponentially growing HeLa cells were transfected with Lipofectamine LTX (Invitrogen, Carlsbad, CA). HEK293-T-cells were grown to 50% confluence and transfected with polyethylenimine (Polysciences, Warrington, PA).

Antibodies and Reagents

Anti-TGN46 antibody was purchased from Novus Biologicals (Littleton, CO); anti-hemagglutinin (HA) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-green fluorescent protein (GFP) antibodies were from Roche Diagnostics (Mannheim, Germany) and Santa Cruz Biotechnology; anti-Myc antibodies were from Cell Signaling Technology (Danvers, MA) and Millipore (Billerica, MA); and anti-glutathione transferase (GST) and anti-His antibodies were from Millipore. Anti-PKD2 and anti-PKD3 antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Anti-PKD2 antibody for immunocytochemistry was purchased from Allele Biotech (San Diego, CA). Alexa 488-, Alexa 594-, and Alexa 647-labeled anti-mouse or anti-rabbit immunoglobulin G were purchased from Invitrogen. Brefeldin A (BFA) was purchased from Biomol (Hamburg, Germany). All other reagents were of the highest grade available.

DNA Constructs

pEGFP-PKD2-WT, D⁶⁹⁵A, S^{706/710}E, Δ CRD, Δ C1a, Δ C1b, Δ PH, and Δ KD have been described previously (Auer *et al.*, 2005). Deletion of amino acids (aa) 1-137 (PKD2- Δ 1-137) was performed in pcDNA3-PKD2-WT (Auer *et al.*, 2005) with a 5' sense primer (5'-gccacctcggaggaaattccagat-3') containing an EcoRI and a 3' antisense primer (5'-cattgtcggatctctgtgaagaacc-3') containing a BamHI site. The fragment was cloned into an EcoRI- and BamHI-digested pEGFP-C2 vector (BD Biosciences Clontech, Erembodegem, Belgium). Deletion of aa 323-368 (PKD2- Δ 323-368) was performed with pcDNA3-PKD2-WT as a template by a splice-overlap polymerase chain reaction (PCR) strategy using *Taq* polymerase (Invitrogen). These primers used resulted in the deletion of aa 323-368: 5'FP (5'-acgatgacagataaagaattgccaccgcc-3'), 3' Δ (5'-aacctcgcagctggtgacgtcattaggagcgggtgg-3'), 5' Δ (5'-ccaccgcctcctaagtactgctcacacgtcgggagggtt-3'), and 3'RP (5'-ggcaactagaaggcacagtcgaggctgat-3'). The final PCR product obtained was then subcloned into a pEGFP-C2 vector to yield pEGFP-PKD2- Δ 323-368. Site-specific mutations within EGFP-PKD2-WT, EGFP-PKD2-D⁶⁹⁵A, EGFP-PKD2- Δ C1b, GFP-PKD1, and GFP-PKD3, resulting in single and double aa substitution mutants (EGFP-PKD2-P¹⁴⁰G, EGFP-PKD2-P²⁷⁵G, EGFP-PKD2-D⁶⁹⁵A-P²⁷⁵G, EGFP-PKD2- Δ C1b-S^{706/710}E, GFP-PKD1-P²⁸⁷G, and GFP-PKD3-P²⁸²G), were performed by a PCR approach

using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). His-tagged C1b domain was generated by amplification of C1b domain from pEGFP-PKD2-WT using a 5' sense primer (5'-cgtacgatcccgccacacctctcatccacagc-3') containing BamHI site and a 3' antisense primer (5'-cgaatcctggtcagctattaggagcgggtggc-3') containing NcoI site. The fragment was cloned into BamHI- and NcoI-digested pRSET-B vector (Invitrogen). Myc-PKD2-WT, D⁶⁹⁵A, and D⁶⁹⁵A-P²⁷⁵G were generated by digestion of enhanced green fluorescent protein (EGFP)-PKD2 constructs with EcoRI and XhoI and then subcloning into pCMV-Tag 3B vector (Stratagene). GST-tagged ARF1 was generated by amplification of full-length human ARF1 from fetal brain cDNA library by PCR, using a 5' sense primer (5'-gcggatccgggaacatcttcccaac-3') containing BamHI site and a 3' antisense primer (5'-gctctgagctcattctggttccggag-3') containing XhoI site. The fragment was cloned into BamHI- and XhoI-digested pGEX-6P1 (GE Healthcare, Uppsala, Sweden) vector. GST-ARF1-T31N and GST-ARF1-Q71L were generated by site-directed mutagenesis. GST-ARF1 Δ 17-Q71L was generated using GST-ARF1 as template with a 5' sense primer (5'-ctactggaattcagcgcactctctggtggcctg-3') containing EcoRI site and a 3' antisense primer (5'-cgataactcagctcactctggtccggagctgattg-3') containing XhoI site. The fragment was cloned into EcoRI- and XhoI-digested pGEX-6P1 vector. GFP-PKD1 and GFP-PKD3 were provided by Dr. Johan van Lint (Katholieke Universiteit Leuven, Leuven, Belgium). ARF1-monomeric red fluorescent protein (mRFP) was a kind gift from Dr. Julie Donaldson (National Institutes of Health, Bethesda, MD). ARF1-Myc was a gift from Dr. Jean Gruenberg (University of Geneva, Geneva, Switzerland). ARF1-T31N-HA, ARF3-T31N-HA, and ARF4-T31N-HA were provided by Dr. Juan S. Bonifacino (National Institutes of Health). ARF5-T31N-HA was provided by Dr. Gwyn Gould (University of Glasgow, Scotland, United Kingdom). pSR α -ARF6-T27N-HA was provided by Dr. Philippe Chavrier (Centre National de la Recherche Scientifique/Institut Curie, Paris, France). GST-ARF6-T27N was generated using pSR α -ARF6-T27N-HA as template with a 5' sense primer (5'-tccccggaattcaggggaaggtgctatcacaatc-3') containing EcoRI site and a 3' antisense primer (5'-cggccctcagctattaagattgtagtaggtaaac-3') containing XhoI site. The fragment was cloned into EcoRI- and XhoI-digested pGEX-6P1 vector. GST-ARF6-WT and GST-ARF6-Q67L were generated by site-directed mutagenesis. Signal sequence from human growth hormone fused to horseradish peroxidase (ss-HRP) was a gift from Dr. Frederic Bard (Institute of Molecular and Cell Biology, Proteos, Singapore). pEGFP-Furin was provided by Dr. Gary Thomas (Vollum Institute, Portland, OR). Vesicular stomatitis virus-G protein (VSV-G)-GFP was provided by Dr. Jennifer Lippincott-Schwartz (National Institutes of Health). All these constructs were confirmed by DNA sequence analysis.

Production and Purification of Recombinant GST-ARF1, GST-ARF6, and His-C1b Proteins

Recombinant proteins were produced and purified as described previously (Cohen *et al.*, 2007). In brief, *Escherichia coli* BL21 host strain was transformed with the pGEX-GST-ARF1, pGEX-GST-ARF6, or pRSET-B-His-C1b expression vectors. Single colonies were inoculated in a 50 ml of liquid broth medium with appropriate antibiotics and cultured overnight at 37°C. Overnight cultures were inoculated (2% inoculum) and grown to OD₆₀₀ of 0.6–0.9 and induced with 1 mM isopropyl β -D-thiogalactoside for 4 h at room temperature. Bacterial cells were pelleted at 4°C, and the pellets were stored at –80°C. Proteins were purified from the bacterial lysates by glutathione-Sepharose 4B beads (for ARF1 and ARF6 constructs) and Nickel-nitrilotriacetic acid agarose (for His-C1b construct) following the manufacturer's instructions.

In Vitro Binding Assay

In vitro binding studies with His-C1b and GST-ARF1 or GST-ARF6 mutants were done as described previously (Cohen *et al.*, 2007). Sepharose-bound GST-ARF1 and ARF6 proteins (~20 μ g) and the purified His-tagged PKD2-C1b domain (~20 μ g) were incubated for 3 h on ice in 0.4 ml of binding buffer (PBS, pH 7.2; 1 mM MgCl₂, 1 mM DTT, 0.2% Triton, and 0.1% Tween 20). Beads (50 μ l) were washed twice with binding buffer (1 ml), and the resulting protein complexes were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoprecipitation and Western Blotting

Immunoprecipitations and Western blotting were performed as described previously (von Blume *et al.*, 2007). Band intensities were quantified using BioProfil BIO-1D software, version 12.04 (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

ARF Pull-down Assay

HEK293-T-cells expressing various PKD isoforms or mutants were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2.5 mM MgCl₂, and protease and phosphatase inhibitor cocktail (Roche Diagnostics), and the extracts were incubated with 50 μ g of GST-ARF1 immobilized on glutathione-Sepharose beads for 2 h at 4°C. Beads were washed extensively, resuspended in Laemmli buffer, and analyzed by SDS-PAGE and Western blotting.

Immunofluorescence Microscopy

HeLa cells were seeded on glass coverslips and transfected the following day. Twenty-four hours after transfection, the cells were fixed with 4% formaldehyde in PBS for 10 min, blocked, and permeabilized with blocking buffer (0.05% saponin and 1% bovine serum albumin in PBS) for 30 min. The coverslips were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature, washed, incubated with secondary antibodies diluted in blocking buffer for 1 h, and then washed and mounted using FluorSave reagent (Calbiochem, Nottingham, United Kingdom). Imaging was performed with a TCS SPE confocal laser scanning microscope (Leica, Wetzlar, Germany), with 405 (Hoechst), 488 (GFP), 532 (mRFP and Alexa Fluor 594), and 635 nm (Alexa Fluor 647) laser lines and a 63× oil objective or with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany), with 488, 543, and 633 nm laser lines and a 63× oil objective. Images were processed using ImageJ (<http://rsbweb.nih.gov/ij/>). The fluorescence intensity of either endogenous PKD2 or overexpressed EGFP-PKD2 within a standardized perinuclear region was measured using the quantification tool in ImageJ.

ss-HRP Secretion Assay

ss-HRP secretion assay was performed as described previously (Bard *et al.*, 2006).

VSV-G Transport Assay

HeLa cells were cotransfected with constructs encoding for a temperature-sensitive viral VSV-G-GFP glycoprotein along with either PKD2 wild type (WT) or mutants and cultured at 39.5°C for 16 h. Then, 100 µg/ml cycloheximide was added before 2-h incubation at 20°C. The cells were then shifted to 32°C in the presence of cycloheximide and subsequently fixed at 15, 45, and 90 min, and processed for immunofluorescence microscopy. Imaging was performed with a TCS SPE confocal laser scanning microscope (Leica) with a 63× oil objective. The fluorescence intensity within a standardized perinuclear region was measured using ImageJ (<http://rsbweb.nih.gov/ij/>).

RNA Interference and Rescue Experiment

siGENOME nontargeting small interfering RNA (siRNA) and siRNA oligonucleotides targeting the 5' untranslated region of PKD2 (5'-cuggguucaugaucgcccgguuu-3') and PKD3 (5'-aaggugaaauccuucguuu-3') were from Dharmacon RNA Technologies (Lafayette, CO). The day before transfection, HeLa cells were plated to ensure 50% confluence on the day of transfection. Knock-down transfections were performed using 20 nM purified siRNA and HiPerfect (QIAGEN, Hilden, Germany). After 24 h of first round of siRNA transfection, HeLa cells were retransfected with siRNA and later followed by transfection with ss-HRP and EGFP expression vector or EGFP-PKD2 vectors (EGFP-PKD2-WT, EGFP-PKD2-ΔC1b, or EGFP-PKD2-P275G) using Lipofectamine LTX (Invitrogen). ss-HRP activity was measured as described previously (Bard *et al.*, 2006).

Statistics

The statistical significance of the difference between means was determined using the two-tailed Student's *t* test. Differences were considered significant at *p* < 0.05.

RESULTS

ARF1 Directly Interacts with PKD2

Because ARFs are prominent Golgi resident proteins and assemble protein complexes at the Golgi, we first we examined whether there was a physical interaction between ARF1 and PKD2. We performed pull-down assays of exogenously expressed GFP-PKD1, EGFP-PKD2, and GFP-PKD3 using GST-ARF1. All three PKD isoforms interacted with GST-ARF1 (Figure 1A). In addition, we performed a pull-down assay with immobilized ARF1 and HeLa cell lysates to detect endogenous PKD2 that is bound to ARF1. As depicted in the Figure 1B, we observed that a significant proportion of endogenous PKD2 (~10% of the input) interacted with immobilized ARF1. This interaction was confirmed by coimmunoprecipitation assays between EGFP-PKD2 and ARF1-Myc expressed in HEK293-T-cells. As shown in Figure 1C, EGFP-PKD2 was detectable in Myc-ARF1 immunoprecipitates, and, vice versa, Myc-ARF1 was found in EGFP-PKD2 immunoprecipitates. The amount of coimmunoprecipitated EGFP-PKD2 and ARF1-Myc was ~3 and 5%, respectively,

compared with the immunoprecipitated ARF1-Myc and EGFP-PKD2.

GTP-bound, active ARF1 recruits coat components and various effectors that constitute the critical machinery of the vesicle budding process at the Golgi (D'Souza-Schorey and Chavrier, 2006). To determine whether the interaction between PKD2 and ARF was dependent on ARF1 activity, we examined the binding of constitutively active and inactive ARF1 mutants. PKD2 binding to the active ARF1 mutant ARF1-Q71L was twofold higher compared with ARF1-WT or the dominant-negative ARF1-T31N mutant (Figure 1D). A similar, twofold increase in binding of PKD2 to ARF1 was obtained when ARF1-Δ17-Q71L was used, a soluble and fully active mutant form of ARF1 (Boehm *et al.*, 2001; Figure 1D). Thus, PKD2 binds to both active and inactive ARF1 mutants but the amount of PKD2 bound to ARF1 was enhanced when ARF1 existed in active confirmation.

PKDs and ARF1 Colocalize at the Golgi Compartment

So far, we have established that PKDs and ARF1 interact physically. It was now important to determine whether there was a spatial relationship between PKD2 and ARF1 at the Golgi compartment. Coexpression of EGFP-PKD2 and ARF1-mRFP in HeLa cells revealed that both proteins colocalized at the TGN as determined by costaining with TGN46, a resident enzyme of the TGN and *trans*-Golgi (Figure 2A). There was also a colocalization of ARF1-mRFP with the other two PKD isoforms, GFP-PKD1 and GFP-PKD3 (Figure 2, B and C). In addition, we observed colocalization of endogenous PKD2 with overexpressed ARF1-mRFP (Figure 2D).

Class I and II ARFs Regulate the TGN Localization of PKD2

To determine whether ARF proteins could play a role in the recruitment of PKD2 to the TGN, we expressed dominant-negative mutants of ARF1, -3, -4, -5, and -6. Expression of these ARF mutants renders the specific endogenous ARFs inactive, presumably by binding to and sequestering ARF-guanine-nucleotide exchange factors (Dascher and Balch, 1994). In the presence of ARF1-T31N, ARF3-T31N, and ARF5-T31N, endogenous PKD2 was largely detectable in the cytosol (Figures 2, F, G, and I, respectively). ARF4-T31N overexpression displayed a moderate effect on the redistribution of endogenous PKD2 from the perinuclear region to the cytosol (Figure 2H). In contrast, upon coexpression of an inactive ARF6-T27N mutant, there was no change in PKD2 localization and PKD2 exhibited the well-known perinuclear localization (Figure 2, J and K). These data were further confirmed by transient overexpression of EGFP-PKD2 and ARF inactive mutants and immunostaining of the TGN compartment (Supplemental Figure S1). These data suggest that the localization of PKD2 to the TGN seems to be mainly regulated by class I and II ARFs. ARF6, the sole member of class III ARFs, seems to have no influence on localization of PKD2 to the TGN.

Interaction of ARF1 with PKD2 Is Mediated by the C1b Domain of PKD2

Having established that PKD2 physically interacts with ARF1, colocalizes with ARF1 at the TGN and that recruitment of PKD2 to the TGN is regulated by class I and II ARFs, we aimed to identify the site in PKD2 that interacts with ARF1. We used various PKD2 deletion mutants, some of which we described previously (Auer *et al.*, 2005; Figure 3A). All mutants exhibited a similar level of expression except for PKD2-Δ1-137 (Figure 3B). The interaction of PKD2 with ARF1 was only abolished upon deletion of the cysteine-rich zinc finger domain. Further analysis revealed that a PKD2

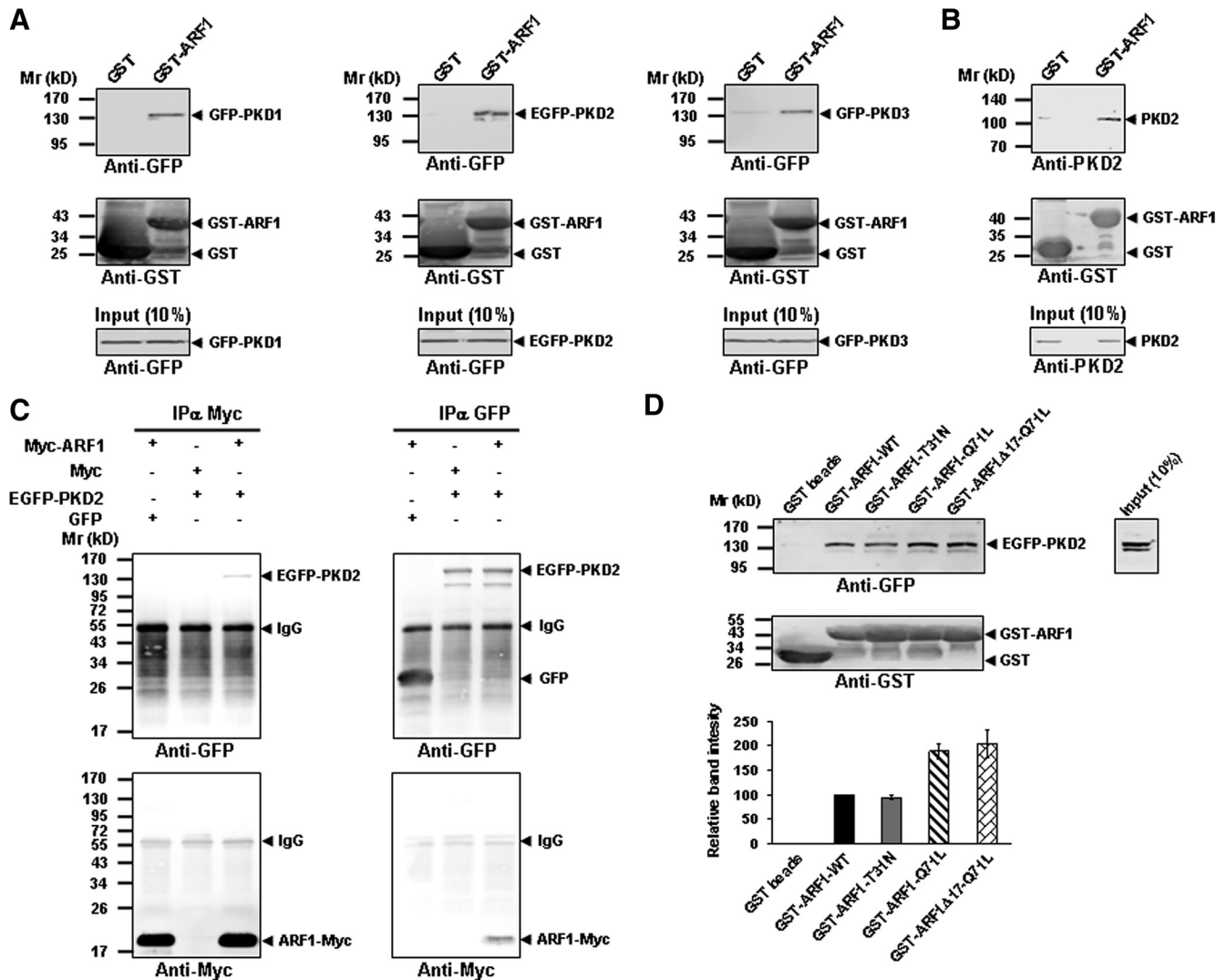


Figure 1. PKD isoforms interact with ARF1. (A) Lysates of HEK293-T-cells expressing GFP-PKD1 or EGFP-PKD2 or GFP-PKD3 were incubated with GST-ARF1 immobilized on glutathione-Sepharose beads, and retained PKD1, -2, or -3 was assessed by Western blotting with GFP antibody. (B) HeLa cell lysate was incubated with GST-ARF1 immobilized on glutathione-Sepharose beads, and retained endogenous PKD2 was assessed by Western blotting with PKD2 antibody. (C) Exogenously expressed PKD2 and ARF1 interact. Myc (lane 2) or Myc-ARF1 (lanes 1 and 3) was coexpressed with EGFP (lane 1) or EGFP-PKD2 (lanes 2 and 3) in HEK293-T-cells. The cells were immunoprecipitated with anti-Myc antibody (top left) or anti-GFP antibody (top right) followed by anti-GFP (top left) or anti-Myc (bottom right) Western blotting, respectively. To verify that each tagged protein was expressed and immunoprecipitated, the Myc and the GFP precipitates were blotted with anti-Myc (bottom left) and anti-GFP, respectively (top right). (D) Lysates of HEK293-T-cells expressing EGFP-PKD2 were incubated with GST-ARF1-WT or mutants immobilized on glutathione-Sepharose beads. Retained PKD2 was assessed by anti-GFP Western blotting. Quantification of the band intensities of PKD2 bound to GST-ARF1-WT or mutants is represented in the bottom panel. Data are means \pm SEM of two independent experiments.

mutant lacking only the C1b domain also failed to interact with ARF1, whereas a PKD2 mutant lacking only the C1a domain could still interact with the GTPase (Figure 3C). Thus, the C1b, but not the C1a domain of PKD2 is critical for its interaction with ARF1. These data were further confirmed by an *in vitro* binding assay using purified recombinant ARF1 and the C1b domain of PKD2. The C1b domain interacted exclusively with ARF1, but not with ARF6 (Figure 3D). Also *in vitro*, binding of the recombinant PKD2-C1b domain to the active ARF1 mutants ARF-Q71L and ARF1- Δ 17-Q71L was increased by 1.5- to 1.75-fold compared with wild-type or dominant-negative ARF1 (Figure 3D). In addition, we observed a similar result when we performed *in vitro* direct binding assays with the recombinant PKD2-C1b domain and purified GST-ARF1 that was preloaded with either guanosine

5'-O-(3-thio)triphosphate (GTP γ S) or guanosine diphosphate (GDP), respectively. PKD2-C1b domain interacted to a similar degree with wild-type ARF1 and GDP-loaded ARF1, but the amount of PKD2-C1b bound to GTP γ S-loaded ARF1 was 1.5-fold higher compared with wild-type and GDP-loaded ARF1 (Supplemental Figure S2).

Targeting of PKD2 to the TGN Requires Its C1b Domain

Next, we examined the role of the C1b domain in the recruitment of PKD2 to ARF1 at the TGN. A PKD2 mutant lacking the C1b domain (PKD2- Δ C1b) failed to colocalize with ARF1. Whereas ARF1-mRFP was largely localized in the perinuclear area corresponding to the Golgi, PKD2- Δ C1b was predominantly detectable in the cytoplasm, but not in the perinuclear area (Figure 3E, bottom, and F; data not shown). This demon-

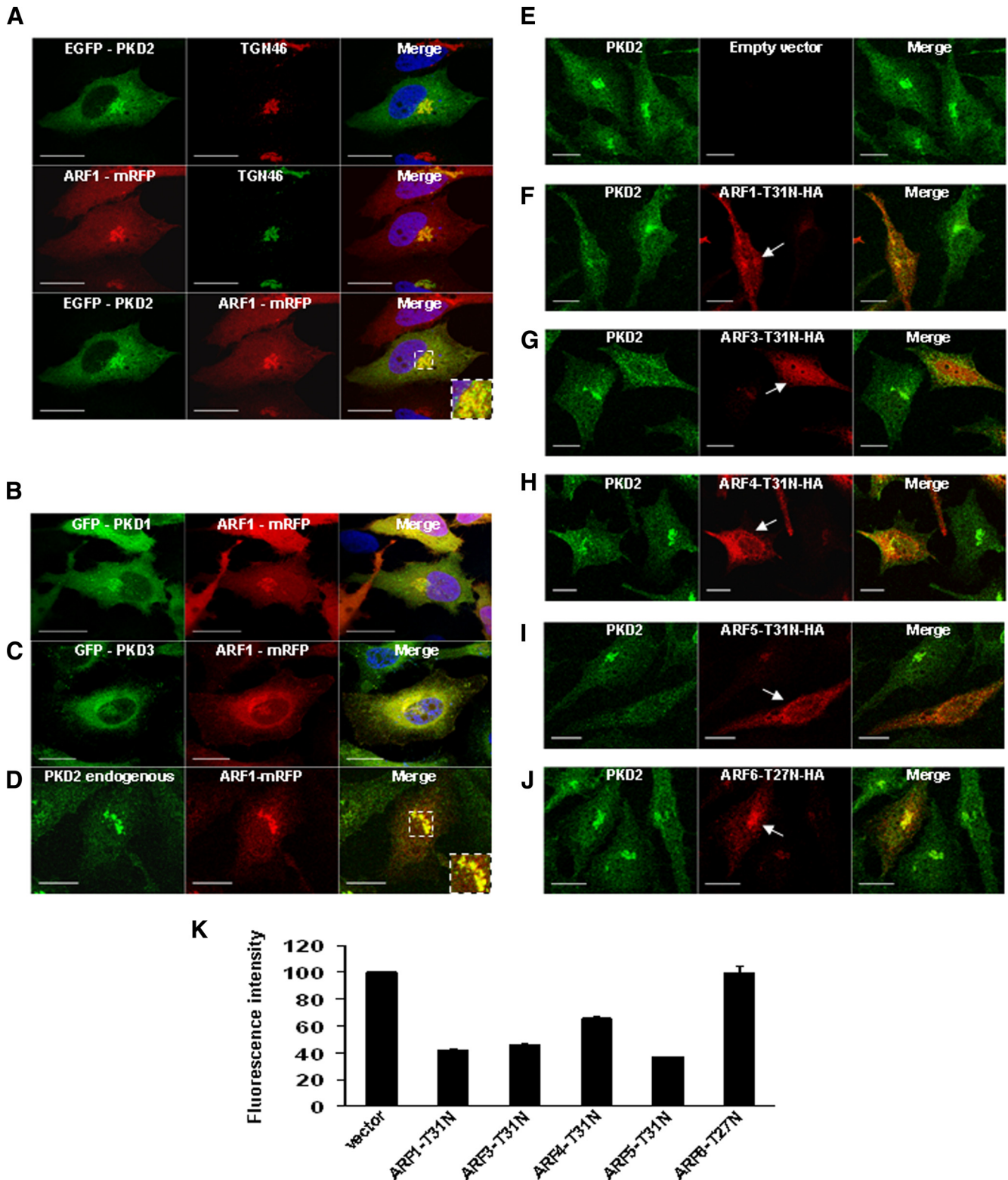


Figure 2. EGFP-PKD2 and ARF1-mRFP colocalize at the Golgi compartment. (A) HeLa cells coexpressing a wild-type EGFP-PKD2 and ARF1-mRFP were fixed followed by anti-TGN46/Alexa 647 immunostaining. The colocalization region is displayed in the zoom area. GFP-PKD1 and GFP-PKD3 colocalize with ARF1-mRFP. (B) HeLa cells coexpressing a wild-type GFP-PKD1 and ARF1-mRFP. (C) HeLa cells coexpressing a wild-type GFP-PKD3 and ARF1-mRFP. (D) HeLa cells expressing ARF1-mRFP were fixed followed by PKD2-antibody/Alexa 488 immunostaining. The colocalization region is displayed in the zoom area. Class I and II ARF proteins specifically regulate the TGN localization of PKD2. HeLa cells overexpressing an empty HA-tag vector (E), ARF1-T31N-HA (F), ARF3-T31N-HA (G), ARF4-T31N-HA (H), ARF5-T31N-HA (I), or ARF6-T27N-HA (J) were fixed followed by HA-antibody/Alexa 594 and PKD2-antibody/Alexa488 immunostaining. Transfected cells are indicated by arrows. Bars, 20 μ m. (K) The histogram shows the quantification of the average fluorescence intensity of endogenous PKD2 for at least 40 cells around the perinuclear area in the cells overexpressing various ARF-inactive mutants. Data are means \pm SEM of two independent experiments.

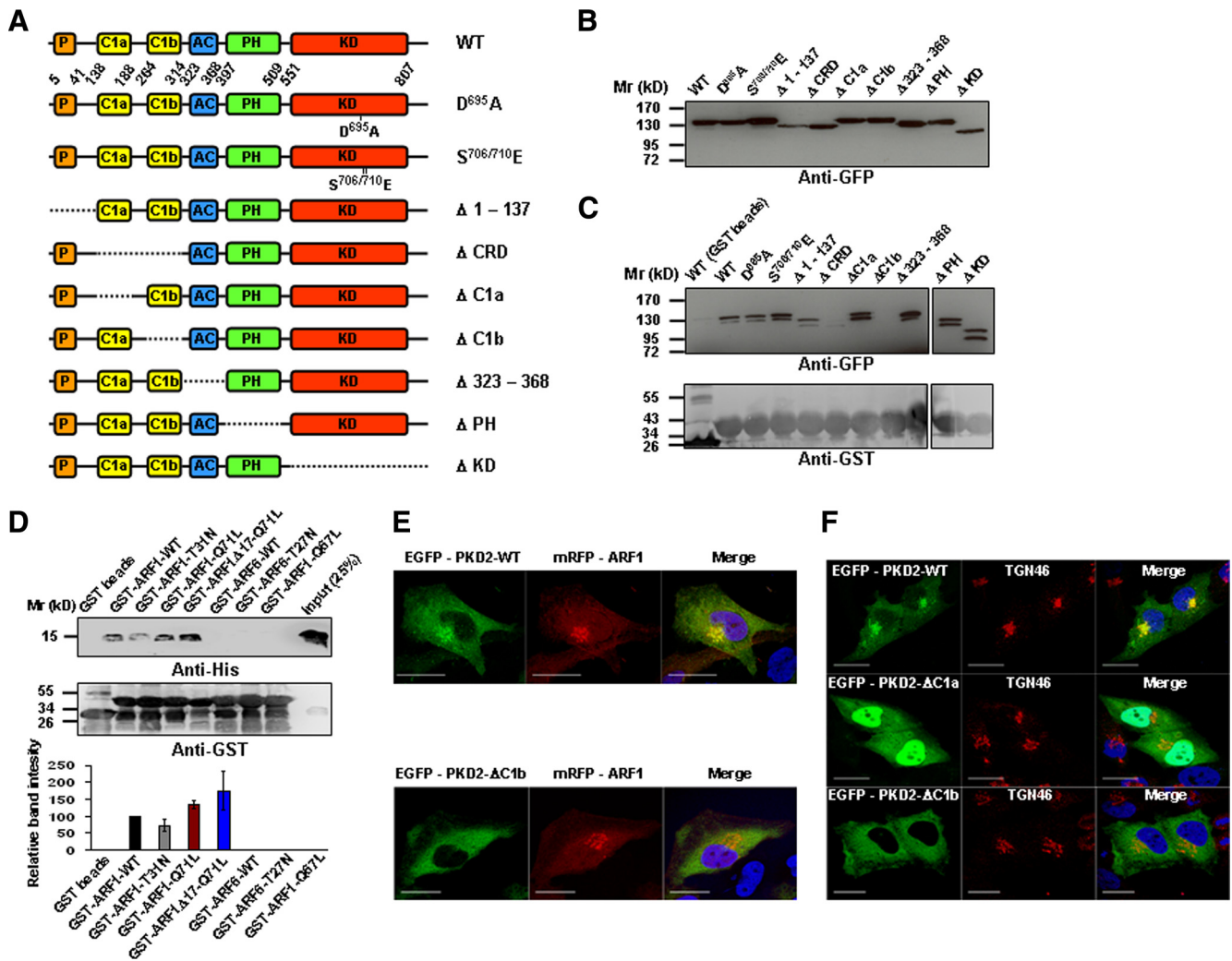


Figure 3. ARF1 specifically interacts with the second cysteine-rich zinc finger domain (C1b) of PKD2 and significance of C1b for Golgi targeting of PKD2. (A) Schematic representation of the PKD2 mutants used in this study. WT, wild-type kinase; D⁶⁹⁵A, kinase dead; S^{706/710}E, constitutively active; Δ1-137, deletion of the first 138 aa; Δ CRD, deletion of the entire cysteine-rich zinc finger domain; ΔC1a, deletion of the first cysteine-rich zinc finger domain; ΔC1b, deletion of the second cysteine-rich zinc finger domain; Δ323-368, deletion of aa 323-368, which includes the acidic domain (AC); ΔPH, deletion of the pleckstrin homology domain; Δ KD, deletion of the kinase domain. (B) HEK293-T-cells expressing EGFP-PKD2 wild type or various mutants as indicated were lysed, and the lysates were incubated with anti-GFP antibody to determine the expression level of the mutants. (C) HEK293-T-cells expressing EGFP-PKD2 WT or various mutants were lysed, and the lysates were incubated with GST-ARF1 immobilized on glutathione-Sepharose beads and retained PKD2-WT and mutants were assessed by Western blotting with GFP antibody, respectively (D) The purified His-tagged C1b domain of PKD2 was incubated with purified GST-ARF1, GST-ARF6, or inactive and active ARF1 and ARF6 mutants immobilized on glutathione-Sepharose beads in an in vitro binding assay. Bound proteins were resolved by Western blotting with anti-His and anti-GST antibodies, respectively. Quantification of the band intensities of His-C1b bound to GST-ARF1 and GST-ARF6 wild type or mutants is represented in the bottom panel. Data are means ± SEM of two independent experiments. HeLa cells coexpressing (E) EGFP-PKD2-WT (top), or EGFP-PKD2-ΔC1b (bottom) and ARF1-mRFP. (F) HeLa cells expressing EGFP-PKD2-WT (top), EGFP-PKD2-ΔC1a (middle), or EGFP-PKD2-ΔC1b (bottom) were fixed followed by anti-TGN46/Alexa-594 immunostaining. Bars, 20 μm.

strates that the C1b domain of PKD2 is critical for its colocalization with ARF1 and the recruitment of the kinase to the TGN.

Previous data show that the C1a domain of PKD1 is necessary for DAG binding (Maeda *et al.*, 2001; Baron and Malhotra, 2002) and that this binding is required for the localization of PKD1 at the TGN. Both PKD2-ΔC1a and PKD2-ΔC1b did not localize to the Golgi (Figure 3F). Thus, Golgi targeting of PKD2 requires both, the C1a domain and the C1b domain of PKD2.

PKD2 exhibits reduced catalytic activity when it lacks the C1b domain (Auer *et al.*, 2005; Supplemental Figure S3A).

Because the catalytic activity of PKD1 is required for its efficient recruitment to the TGN (Maeda *et al.*, 2001), we compensated the reduced catalytic activity of PKD2-ΔC1b by introducing phosphomimetic glutamic acid residues at the critical serine residues within the activation loop of the kinase. The PKD2-ΔC1b-S^{706/710}E mutant exhibited an elevated level of catalytic activity comparable to wild-type PKD2 that was well above that of catalytically inactive PKD2 (Supplemental Figure S3A). However, the PKD2-ΔC1b-S^{706/710}E mutant neither interacted (Supplemental Figure S3B) nor colocalized with ARF1 (Supplemental Figure S3C) and did not localize to the TGN (Supplemental Figure S3D), demonstrating that the lack of catalytic

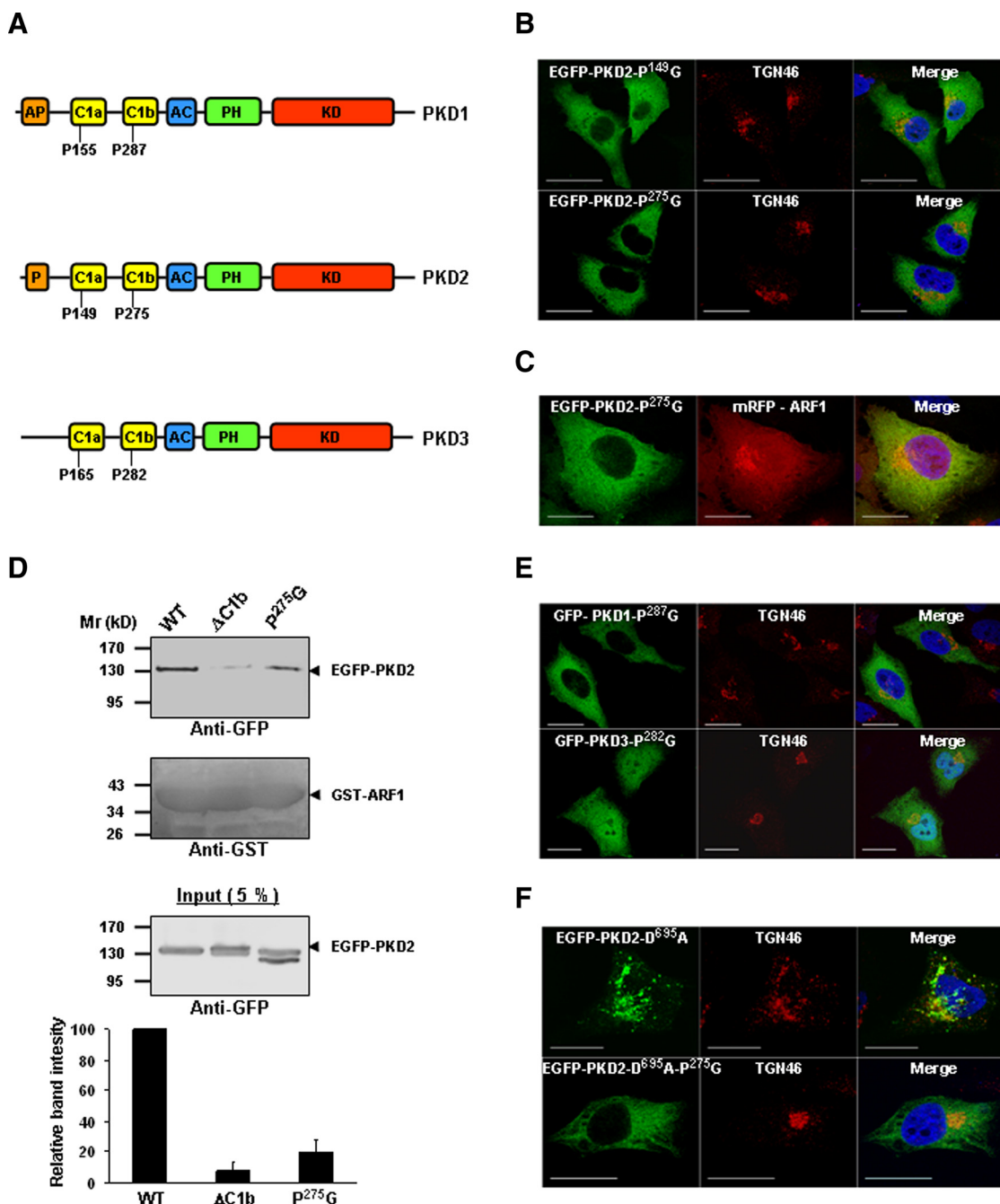


Figure 4. Pro275 in the C1b domain of PKD2 is critical for TGN localization and ARF1 binding. (A) Schematic representation of the critical proline residues in the CRD of PKD1 and corresponding Pro sites in PKD2 and PKD3. (B) HeLa cells expressing EGFP-PKD2-P¹⁴⁹G (top) or EGFP-PKD2-P²⁷⁵G (bottom) were fixed followed by anti-TGN46/Alexa 594 immunostaining. (C) HeLa cells coexpressing EGFP-PKD2-P²⁷⁵G and ARF1-mRFP. (D) HEK293-T-cells expressing EGFP-PKD2-WT, EGFP-PKD2-ΔC1b, or EGFP-PKD2-P²⁷⁵G were lysed, and the lysates were incubated with GST-ARF1 immobilized on glutathione-Sepharose beads. Bound PKD2 wild type or mutants were assessed by anti-GFP Western blotting (top). Quantification of the band intensities of wild-type PKD2 or mutants bound to GST-ARF1 is represented in the bottom panel. Data are means \pm SEM of four independent experiments. (E) HeLa cells expressing EGFP-PKD1-P²⁸⁷G (top) or EGFP-PKD3-P²⁸²G (bottom) were fixed followed by anti-TGN46/Alexa-594 immunostaining. (F) HeLa cells expressing EGFP-PKD2-D⁶⁹⁵A (top) or EGFP-PKD2-D⁶⁹⁵A-P²⁷⁵G (bottom) were fixed followed by anti-TGN46/Alexa-594 immunostaining. Bars, 20 μ m.

activity was not responsible for the lack of Golgi targeting of PKD2-ΔC1b.

Pro275 within the C1b Domain Is Critical for ARF1 Binding and TGN Targeting of PKD2

Next, we wanted to identify the region within the C1b domain of PKD2 required for the interaction with ARF1.

There are critical Pro residues within the zinc finger domains that are conserved in all the three PKD isoforms (Figure 4A). Pro155 within the C1a domain of PKD1 is necessary for the binding of the kinase to DAG and for its localization at the TGN (Maeda *et al.*, 2001; Baron and Malhotra, 2002). Pro155 and 287 within the C1a and C1b domain of PKD1, respectively, have been described as important

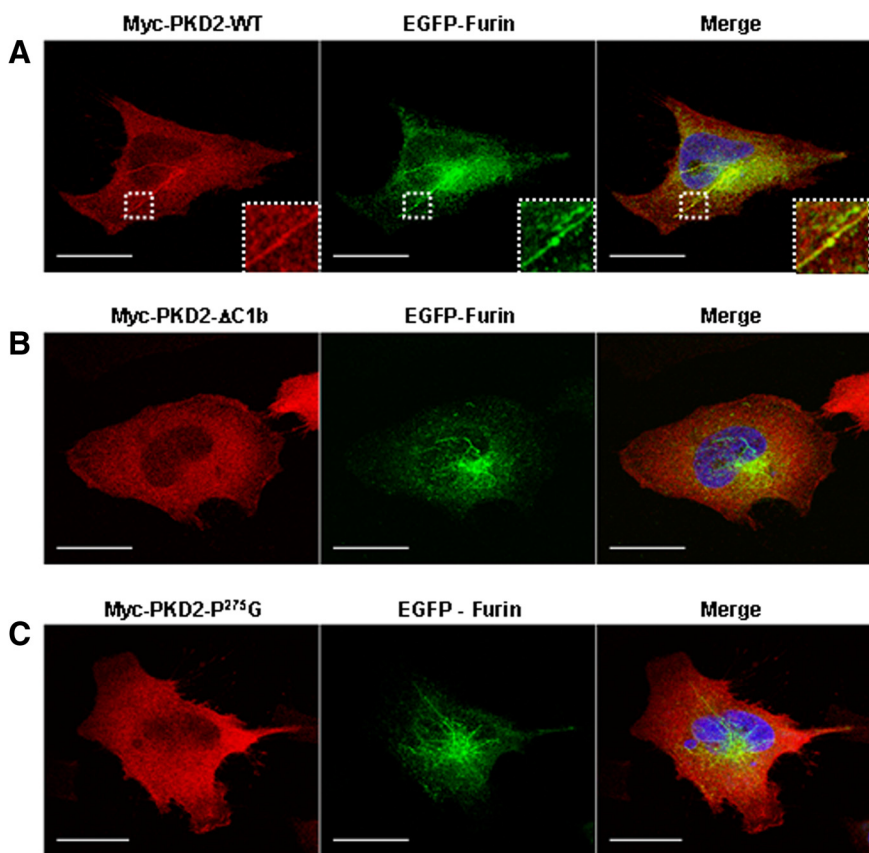


Figure 5. Wild-type PKD2, but not PKD2- Δ C1b or PKD2-P275G, redistributes into Golgi tubules induced by short-term BFA treatment. HeLa cells coexpressing EGFP-furin and wild-type Myc-PKD2 (A), Myc-PKD2 Δ C1b (B), or Myc-PKD2-P275G (C) were treated with BFA (final concentration, 5 μ g/ml) for 5 min and then fixed by anti-Myc/Alexa 594 immunostaining. The colocalization region of wild-type Myc-PKD2 and EGFP-furin is displayed in the zoom area. Bars, 20 μ m.

residues for plasma membrane localization of PKD1 in lymphocytes (Spitaler *et al.*, 2006).

Mutation of the conserved Pro within the C1a domain of PKD2 (EGFP-PKD2-P¹⁴⁹G) abolished the perinuclear, TGN localization of the kinase as determined by TGN46 costaining and exhibited a largely cytosolic localization (Figure 4B, top), similar to previous studies using the corresponding PKD1-Pro¹⁵⁵G mutant (Maeda *et al.*, 2001). Interestingly, the exchange of Pro at position 275 in the C1b domain of PKD2 to Gly also resulted in a predominantly cytosolic localization of this mutant (Figure 4B, bottom). In addition, EGFP-PKD2-P²⁷⁵G failed to colocalize with ARF1 (Figure 4C). The interaction of EGFP-PKD2-P²⁷⁵G with ARF1 was also reduced by 80% compared with wild-type PKD2 in a pull-down assay (Figure 4D). Occasionally, we observed a double band for PKD2- Δ C1b and PKD2-P²⁷⁵G. This is most likely due to C-terminal degradation of the protein. The critical role of Pro275 in the binding of PKD2 to ARF1 was further confirmed in an *in vivo* coimmunoprecipitation assay. The amount of PKD2 detectable in ARF1-immunoprecipitates was reduced by 80% when the Pro275 was mutated compared with the wild-type PKD2 (Supplemental Figure S4). These data indicate that Pro275 in the C1b domain is critical for binding ARF1 and also critical for recruiting PKD2 to the TGN. The mutation of the corresponding conserved Pro residues in PKD1 and PKD3, Pro287 and Pro282, respectively, also resulted in a complete loss of TGN localization of these PKD isoforms (Figure 4E).

Expression of a catalytically inactive PKD1 or PKD2 leads to tubulation of the TGN and thereby inhibits vesicle shedding and protein transport (Liljedahl *et al.*, 2001; Figure 4F, top). A P¹⁵⁵G mutation in the C1a domain of kinase dead PKD1 prevents post-Golgi tubulation normally induced by

catalytically inactive PKD1 (Maeda *et al.*, 2001). Expression of a kinase dead EGFP-PKD2 additionally mutated at Pro²⁷⁵G in the C1b domain of PKD2 (EGFP-PKD2-D⁶⁹⁵A-P²⁷⁵G) in HeLa cells abolished post-Golgi tubulation normally observed upon expression of the catalytically inactive mutant (Figure 4F, bottom).

Short-term BFA treatment induces tubulation of the early Golgi cisternae, TGN, and the endosomes (Lippincott-Schwartz *et al.*, 1991). Under these conditions, PKD1 does not dissociate from the TGN because DAG can still serve as the receptor for PKD1 at the TGN. However, PKD1 redistributes into the BFA-induced tubules positive for TGN46 and furin, a recycling TGN-plasma membrane-endosomal marker (Maeda *et al.*, 2001). On short-term (5-min) BFA treatment of HeLa cells, wild-type PKD2 also redistributed into BFA-induced, furin-positive tubules in line with the data obtained with PKD1 (Figure 5A). However, both EGFP-PKD2- Δ C1b and EGFP-PKD2-P²⁷⁵G did not exhibit any redistribution to BFA-induced tubules (Figure 5, B and C). This further proves that these mutants are not recruited to the TGN in the first place and can therefore be not redistributed into furin-positive tubules upon BFA treatment.

Role of the C1b Domain and Pro275 for PKD2-mediated Protein Transport from the TGN to the Plasma Membrane

Next, we asked whether Pro275 in PKD2 was also critical for the PKD2-mediated transit of proteins from the TGN to the plasma membrane. We used ss-HRP as a marker for vesicular transport. ss-HRP is a secretory marker in which the signal sequence from human growth hormone (hGH) is fused to HRP (Connolly *et al.*, 1994). When this construct is expressed in cells, it is synthesized and transported along the secretory pathway. Because it has the characteristic sig-

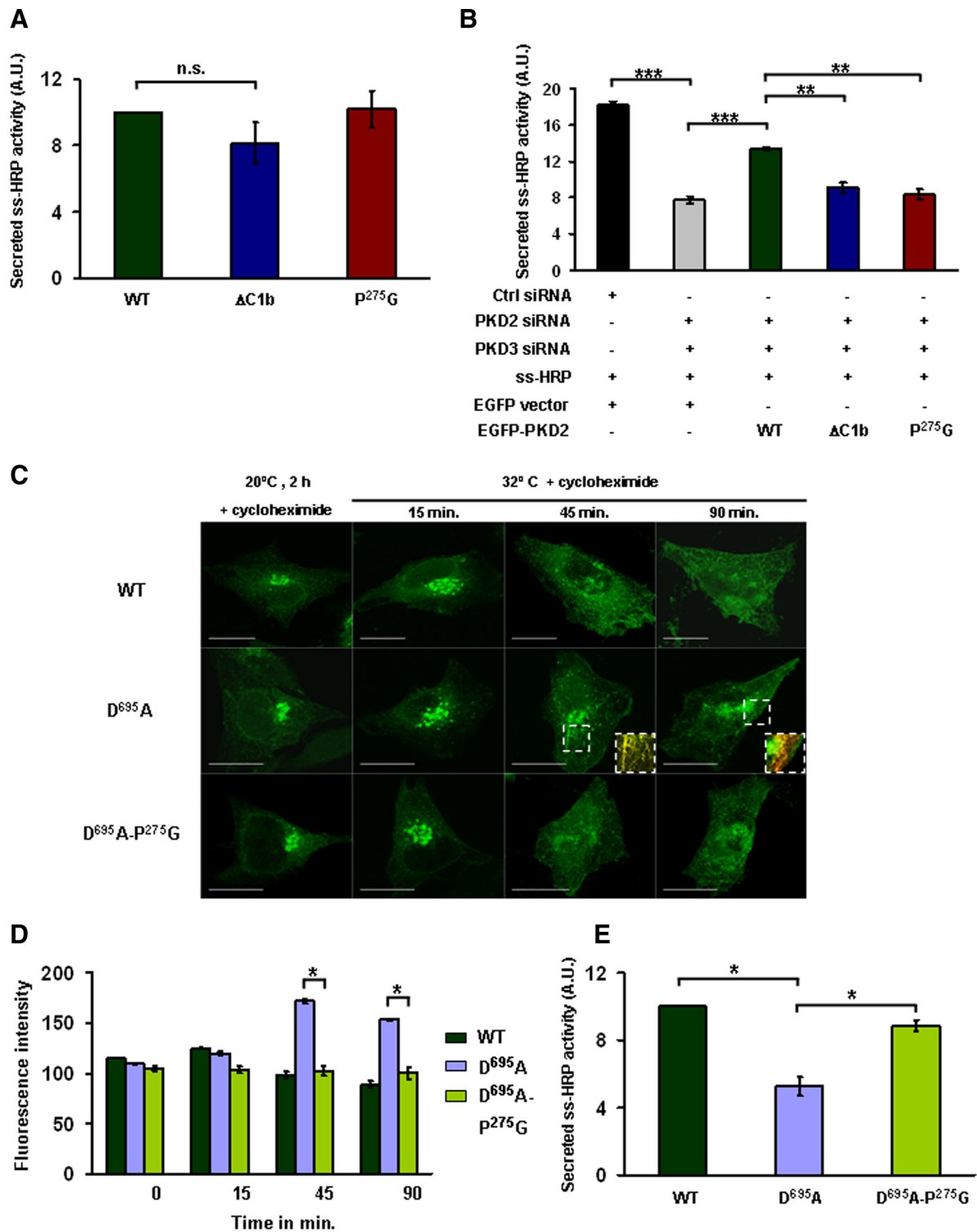


Figure 6. Significance of C1b and Pro275 in PKD2-mediated secretory transport. (A) ss-HRP and EGFP-PKD2-WT (WT) or EGFP-PKD2-ΔC1b (ΔC1b), and EGFP-PKD2-P²⁷⁵G (P²⁷⁵G) were cotransfected in HeLa cells. Twenty-four hours after transfection, HRP activity secreted in the medium was measured by chemiluminescence. Bars represent the means \pm SEM of three independent experiments of HRP activity in the medium normalized to intracellular ss-HRP expression levels. (B) HeLa cells depleted of PKD2 and PKD3 were subsequently cotransfected with ss-HRP and EGFP or EGFP-PKD2-WT (WT), EGFP-PKD2-ΔC1b (ΔC1b), or EGFP-PKD2-P²⁷⁵G (P²⁷⁵G) expression plasmids. Bars represent the means \pm SEM of four independent experiments of HRP activity in the medium normalized to intracellular ss-HRP expression levels. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (C) Only kinase dead PKD2-D⁶⁹⁵A, but not PKD2-D⁶⁹⁵A-P²⁷⁵G blocks VSV-G-GFP transport. HeLa cells coexpressing the secretory marker protein VSV-G-GFP and Myc-PKD2-WT (WT), Myc-PKD2-D⁶⁹⁵A (D⁶⁹⁵A), and Myc-PKD2-D⁶⁹⁵A-P²⁷⁵G (D⁶⁹⁵A-P²⁷⁵G) were grown at 39.5°C overnight. On accumulation of VSV-G-GFP in the Golgi at 20°C for 2 h, cells were then incubated at 32°C for different times in the presence of cycloheximide to permit the transport of VSV-G-GFP from the Golgi along the secretory pathway. The cells were then fixed followed by anti-Myc/Alexa-594 immunostaining to identify double-positive cells expressing

nal sequence, it is exported to the plasma membrane and secreted into the supernatant of cells. The secreted protein can be quantified by measuring the chemiluminescence signal obtained by adding enhanced chemiluminescence substrate (Bard *et al.*, 2006). In cells expressing wild-type PKD2, ss-HRP was efficiently secreted into the supernatant (Figure 6A). The amount of ss-HRP secreted was not significantly affected in cells expressing PKD2- Δ C1b or PKD2-P²⁷⁵G (Figure 6A) as these mutants do not localize to the Golgi, do not interact with ARF1, and are unlikely to compete with endogenous PKDs for the interaction with ARF1 at the TGN. Therefore, endogenous PKDs can act normally at the TGN and regulate protein transport. A similar secretory phenotype was observed with the green fluorescent protein-tagged ts045 mutant VSV-G-GFP, a well-characterized exocytic marker (Bergmann, 1989). In cells expressing wild-type PKD2, VSV-G was also efficiently transported to the plasma membrane, and the transport of VSV-G was not affected in cells expressing PKD2- Δ C1b or PKD2-P²⁷⁵G (data not shown).

To further determine whether PKD2- Δ C1b and PKD2-P²⁷⁵G had any functional activity at the TGN, we expressed these mutants in HeLa cells that were depleted of endogenous PKDs by specific siRNAs. HeLa cells express predominantly PKD2 and PKD3 (Bossard *et al.*, 2007). The siRNA-mediated knockdown of PKD2 and PKD3 in HeLa cells resulted in efficient depletion (70–80%) of both PKD2 and PKD3 protein (Supplemental Figure 5A). After siRNA knockdown of PKD2 and PKD3, HeLa cells were transfected with a plasmid encoding ss-HRP together with an EGFP-expression vector, wild-type PKD2, EGFP-PKD2- Δ C1b, or EGFP-PKD2-P²⁷⁵G. The expression level of the various EGFP-tagged PKD2 constructs was comparable (Supplemental Figure 5B). Knockdown of PKD2 and PKD3 resulted in a substantial decrease of HRP secretion in line with previous data (Bossard *et al.*, 2007; (Figure 6B). Expression of wild-type PKD2 in HeLa cells depleted of endogenous PKD2 and PKD3 rescued HRP secretion. In marked contrast, neither expression of EGFP-PKD2- Δ C1b nor of EGFP-PKD2-P²⁷⁵G resulted in a significant increase in secreted HRP and therefore failed to rescue the secretory block imposed by the PKD2/PKD3 knockdown (Figure 6B). These rescue experiments demonstrate the critical role of the C1b domain and particularly Pro275 in PKD2 for TGN targeting of the kinase and subsequently PKD2 function at the TGN, i.e., the regulation of protein transport from the TGN to the plasma membrane.

Having demonstrated that PKD2- Δ C1b and PKD2-P²⁷⁵G cannot rescue the phenotype induced by knockdown of PKDs due to a lack of Golgi localization, these mutants should also modify the effect of kinase dead PKD2. In cells expressing kinase-dead PKD2 (PKD2-D⁶⁹⁵A) that localizes

to the Golgi and can compete with endogenous PKDs, VSV-G accumulated within TGN tubules that were positive for PKD2-D⁶⁹⁵A, and protein transport was substantially interrupted (Figure 6C), in line with previous data (Liljedahl *et al.*, 2001, Yeaman *et al.*, 2004). Fluorescence quantification confirmed that cells expressing PKD2-D⁶⁹⁵A exhibited a 1.7-fold increase in VSV-G protein at the Golgi region (Figure 6D). Interestingly, cells expressing a PKD2-D⁶⁹⁵A-P²⁷⁵G double mutant did not exhibit an increase in VSV-G protein at the Golgi region, indicating that this mutant failed to block VSV-G transport. This shows that the Pro275 mutation prevents Golgi targeting of the kinase dead, dominant-negative-acting kinase and consequently its dominant-negative effect on endogenous PKDs and their role in vesicle shedding and protein transport from the TGN (Figure 6, C and D). This finding further confirmed that Pro275 in the PKD2-C1b domain is crucial for the function of the kinase at the TGN. A similar secretory phenotype was observed with HRP protein secretion. The P²⁷⁵G mutation also reversed the inhibitory effect of PKD2-D⁶⁹⁵A on HRP secretion (Figure 6E).

DISCUSSION

PKDs play a major role in regulating protein transport from the TGN to the plasma membrane. There has been a substantial amount of work elucidating the mechanisms by which PKDs regulate vesicle shedding. A local pool of DAG plays a major role in the recruitment of PKD to the TGN (Bard and Malhotra, 2006; Bossard *et al.*, 2007). Active PKD phosphorylates PI4KIII β and CERT, the two important PKD substrates identified so far at the TGN. The PKD-mediated phosphorylation of PI4KIII β and CERT is critical in regulating the cross-talk between the membrane lipid biogenesis and protein secretion. This in turn regulates the maintenance of local DAG and PKD tethering to the TGN, which ultimately ensures a controlled vesicular transport process from the TGN (Hausser *et al.*, 2005; Bard and Malhotra, 2006; Fugmann *et al.*, 2007). Previous work demonstrated that the C1a domain of PKDs is crucial for the localization of PKDs at the TGN via binding of DAG (Maeda *et al.*, 2001). However, the precise mechanisms how PKDs are recruited to the TGN are as yet less clear.

The small GTPases of the ADP-ribosylation factor family are also master regulators of the structure and function of the Golgi complex. Among the three classes of the ARF family, class I and II were reported to exert their function at the Golgi compartment. Active ARF1 recruits COPI coats which interact with the bona fide cargo proteins and generate functional vesicles that operate in the intra-Golgi and Golgi-endoplasmic reticulum retrograde trafficking zones of the membrane trafficking process (Orci *et al.*, 1993). In addition, ARF1 was also shown to be one of the major components of the sorting machinery and is involved in controlling multiple TGN exit pathways (De Matteis and Luini, 2008). Many of the effectors and regulators of ARF1 play an important role in the formation and scission of vesicles destined for distinct compartments of the cell.

Here, we demonstrate that PKD2 specifically and directly interacts with ARF1. Binding of PKD2 to ARF1 is affected by the nature of the nucleotide bound to the GTPase and the association of PKD2 with ARF1 is enhanced when the GTPase exists in active confirmation. However, there is also an interaction between PKD2 and inactive ARF1. This is in line with previous reports that described the association of ARF1 with effector proteins such as the HIV Nef protein and the μ subunit of the adaptor protein complex AP-4 that

Figure 6 (cont). VSV-G-GFP and wild-type PKD2 (top) or mutant PKD2 (middle and bottom). VSV-G-GFP trapped in post-Golgi tubules upon expression of PKD2 D⁶⁹⁵A is shown as merged image insert in the middle panel. Bars, 20 μ m. (D) Histogram shows the quantification of the average fluorescence intensity of VSV-G-GFP for at least 40 cells around the perinuclear area in the cells coexpressing wild-type PKD2 or various mutants. Data are means \pm SEM of two independent experiments. * $p < 0.05$. (E) ss-HRP and EGFP-PKD2-D⁶⁹⁵A (D⁶⁹⁵A) and EGFP-PKD2-D⁶⁹⁵A-P²⁷⁵G (D⁶⁹⁵A-P²⁷⁵G) were cotransfected in HeLa cells. Twenty-four hours after transfection, HRP activity secreted in the medium was measured by chemiluminescence. Bars represent the means \pm SEM of three independent experiments of HRP activity in the medium normalized to intracellular ss-HRP expression levels. * $p < 0.05$.

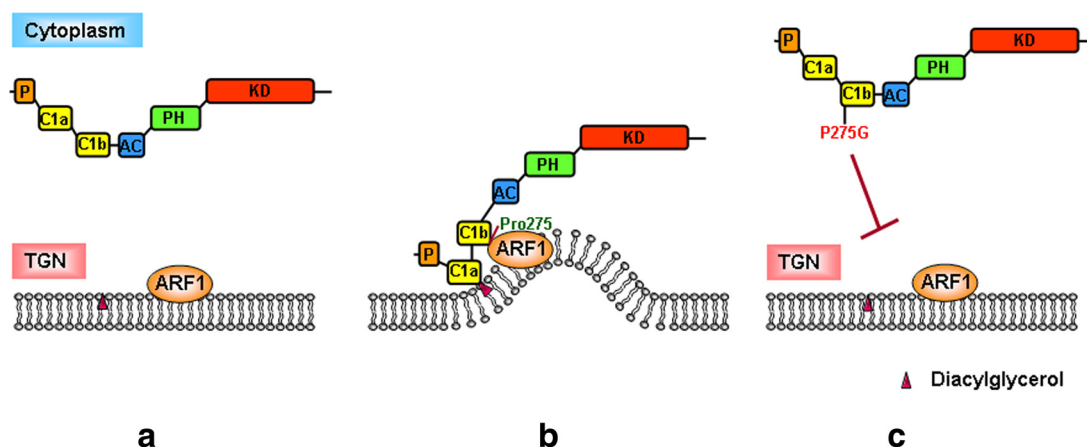


Figure 7. Model depicting PKD2 recruitment and function at the TGN. PKD2 localized in the cytoplasm (a) is recruited to the TGN by binding to ARF1 via Pro275 within the C1b domain. This results in further positioning by interaction with DAG via C1a domain and thereby accomplishes vesicle shedding (b); a Pro275 mutation renders PKD2 to be cytosolic by preventing its interaction with ARF1 and thereby its localization to the TGN (c), which ultimately blocks protein transport from the TGN to the plasma membrane.

interact with ARF1 independently of the nucleotide status of the GTPase (Boehm et al., 2001; Faure et al., 2004).

Expression of dominant-negative mutants of ARFs that are locked in the GDP conformation serve as an important tool in studying the effect of different ARF isoforms on the subcellular localization of various effector proteins. We found that the localization of PKD2 at the TGN was regulated by class I and II ARFs, which are known to play indistinguishable roles at the Golgi complex.

ARF1 and PKD2 not only interact *in vitro* and *in vivo* but also colocalize at the TGN as demonstrated by immunocytochemistry. In addition, the interaction of PKD2 with ARF1 is specifically mediated by its C1b domain and Pro275 within this domain is the central amino acid required for the ARF1-PKD2 interaction. A PKD2 mutant lacking the C1b domain or exhibiting a P²⁷⁵G exchange not only fails to interact with ARF1 but also does not localize to the Golgi and is largely localized in the cytoplasm. This points to a crucial role for the interaction of PKD2 with ARF1 to target PKD2 to the Golgi compartment. Our study shows that ARF1 functions as an important receptor for PKD in addition to local pool of DAG at the TGN. This also explains the requirement of an additional mechanism to target PKD to the TGN, despite the fact that DAG is present at various cellular locations. In line with this conclusion, our data further demonstrate that the loss of the C1b domain or the P²⁷⁵G exchange abolish the functional activity of PKD2 at the Golgi compartment. Both mutants cannot rescue the block of protein transport from the TGN to the plasma membrane induced by siRNA-mediated knockdown of endogenous PKD2 and -3 in HeLa cells. Furthermore, a lack of the C1b domain or a P²⁷⁵G mutation also abolished the dominant-negative effect of kinase dead PKD2 on protein transport from the TGN.

In conclusion, these data suggest a novel model in which the localization of PKD2 to the TGN requires both, the C1a and C1b domain: ARF1 recruits PKD2 from the cytoplasm (Figure 7a) to the Golgi apparatus via binding of Pro275 in the C1b domain of PKD2. The kinase is then anchored at the TGN by interacting with DAG in the membrane via its C1a domain. Both processes are required to accomplish vesicle shedding (Figure 7b). When the C1b domain is deleted or the critical Pro275 in PKD2 is mutated, ARF1 binding is impaired resulting in cytoplasmic localization of PKD2 and

loss of function at the Golgi (Figure 7c). These data provide the first link between the “classical” machinery regulating protein transport at the Golgi compartment, namely, ARF proteins, and PKDs and demonstrate that the direct interaction of both is crucial for efficient protein transport from the TGN to the plasma membrane.

ACKNOWLEDGMENTS

We are thankful to Yuichi Wakana, Sandra Mitrovic, Juan Duran, Josse Van Galen and Kota Saito of the Malhotra lab for useful discussions. We thank Timo Zimmermann and Frank Dolp for support with the TCS SPE confocal microscope and LSM510 confocal laser scanning microscope. G.V.P is a fellow of the GRK1041 and International Graduate School in Molecular Medicine, Ulm. T. S. is supported by Deutsche Forschungsgemeinschaft (SFB 518/B3 and A15 and GRK1041).

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