Protein kinase D (PKD) controls protein traffic from the trans-Golgi network (TGN) to the plasma membrane of epithelial cells in an isoform-specific manner. However, whether the different PKD isoforms could be selectively regulating the traffic of their specific substrates remains unexplored. We identified the C terminus of the different PKDs that constitutes a postsynaptic density-95/discs large/zonula occludens-1 (PDZ)-binding motif in PKD1 and PKD2, but not in PKD3, to be responsible for the differential control of kinase D-interacting substrate of 220-kDa (Kidins220) surface localization, a neural membrane protein identified as the first sub-strate of PKD1. A kinase-inactive mutant of PKD3 is only able to alter the localization of Kidins220 at the plasma membrane when its C terminus has been substituted by the PDZ-binding motif of PKD1 or PKD2. This isoform-specific regulation of Kidins220 transport might not due to differences among kinase activity or substrate selectivity of the PKD isoenzymes but more to the adaptors bound to their unique C terminus. Furthermore, by mutating the auto-phosphorylation site Ser916, located at the critical position -2 of the PDZ-binding domain within PKD1, or by phorbol ester stimulation, we demonstrate that the phosphorylation of this residue is crucial for Kidins220-regulated transport. We also discovered that Ser916 trans-phosphorylation takes place among PKD1 molecules. Finally, we demonstrate that PKD1 association to intracellular membranes is critical to control Kidins220 traffic. Our findings reveal the molecular mechanism by which PKD localization and activity control the traffic of Kidins220, most likely by modulating the recruitment of PDZ proteins in an isoform-specific and phosphorylation-dependent manner.

Protein kinase D1 (PKD1)\(^5\) (1), also known as PKC\(\mu\) (2), belongs to a novel family of diacylglycerol (DAG)-stimulated Ser/Thr kinases, composed of two more members PKD2 (3) and PKD3 (4) (reviewed in Refs. 5–7). PKDs contain several well characterized domains, including two cysteine-rich repeats (C1a and C1b) that constitute a C1 domain (CR), a pleckstrin homology domain, and a catalytic domain at the C terminus. The CR domain binds DAG and phorbol esters with high affinity (1, 8) and is involved in the association of PKD1 to cellular membranes such as the plasma membrane and the trans-Golgi network (TGN) (9–12). The pleckstrin homology domain is an autoinhibitory domain that regulates the activity of this kinase (13). PKD is activated by the phosphorylation of two activation loop sites within the catalytic domain through a protein kinase C (PKC)-dependent pathway, which stabilizes the enzyme in an active conformation (14, 15). Activated PKD1 autophosphorylates at Ser916 present at the very C terminus, and this phosphorylation event is frequently used to determine the activation state of this kinase (16–18).

PKD has been shown to participate in many cellular processes, such as cell survival, proliferation, and invasion (5–7), but the regulation of protein transport represents one of the most studied functions of this kinase. PKD1 was first implicated in the control of constitutive membrane protein secretion from the TGN to the cell surface in HeLa cells, by regulating the fission of vesicles originating from this compartment (19–21). It has been also reported that PKD isoforms differentially regulate the exit of vesicle-derived exocytic carriers from the TGN to the basolateral membrane in polarized epithelial cells, supporting the idea that each isoform could be controlling the transport of a subset of vesicular cargos to the plasma membrane, probably by recognizing them as specific substrates (22). Very recently, phosphatidylinositol 4-kinase III\(\beta\), a Golgi membrane-localized lipid kinase that plays a key role in the structure and function of this organelle, has been found to be a physiological substrate of PKD. All three PKD isoforms, PKD1, PKD2, and to a lesser extent PKD3, phosphorylate phosphatidylinositol 4-kinase III\(\beta\) and stimulate its kinase activity, enhancing vesicular stomatitis virus G-protein transport to the plasma membrane (23). This novel discovery has helped to reveal some of the molecular events that enable transport-carrier formation at the TGN. However, the molecular mechanisms by which the different PKD isoforms could be selectively regulating the traffic of some of their specific substrates to the cell surface remain widely unexplored.

A few years ago we cloned kinase D-interacting substrate of 220 kDa (Kidins220) from PC12 cells (24), and we identified this novel protein as the first physiological substrate for PKD1. Kidins220, also known as ARMS (ankyrin-rich membrane spanning; see Ref. 25), is an integral...
plasma membrane protein predominantly expressed in brain and neurons that presents a polarized location in neural cells, concentrating at the tip of extending neurites and in plasma membrane micro-domains corresponding to lipid rafts (18, 24). The primary amino acid sequence of Kidins220 reveals 11 ankyrin repeats, 4 trans-membrane domains, a proline-rich region, a sterile-α motif (or SAM) domain, and a type I postsynaptic density-95/discs large/zonula occludens-1 (PDZ)-binding motif at the very C terminus (24, 25).

Consensus PDZ-binding domains are sequences of four amino acids present at the C terminus of many membrane proteins (ion channels, transporters and receptors) that bind to PDZ proteins (26). The consensus sequence of type I PDZ-binding motifs is $X^3$-(Ser/Thr)$^2X^2$-$\phi\phi$, where $X$ is any amino acid, $\phi$ is a hydrophobic amino acid and $-3, -2, -1$, and 0 refer to the position starting from the C-terminal residue. Amino acids at position 0 and $-2$ are the critical determinants for the binding of specific type I PDZ domains to these ligands (26). PDZ domains are found as single or multiple repeats in nearly 400 unrelated PDZ proteins (26). By binding PDZ ligands, PDZ proteins play a crucial role in cellular polarization as well as in the different processes that control the correct surface expression and function of membrane proteins, including scaffolding and/or retention at the plasma membrane, endocytosis, and asymmetrical delivery from the TGN to the different domains at the cell surface (27–29).

In this study, we report that PKD1 and also PKD2, but not PKD3, specifically regulate the surface localization of Kidins220 in cells of nervous origin such as primary cortical neurons and PC12 cells. Importantly, the differential control exerted by PKD isoforms relies on a newly identified domain, a type I PDZ-binding motif present at the very C-terminal end of PKD1 and PKD2 that is absent in the PKD3 sequence. Importantly, we demonstrate that autophosphorylation or homologous transphosphorylation of the critical Ser$^{916}$ at position −2 within this motif in PKD1 is crucial for Kidins220 correct localization at the plasma membrane, likely by modulating the recruitment of PDZ proteins at the TGN. Furthermore, we show by deleting the CR domain that PKD1 association with intracellular membranes is critical to control Kidins220 traffic. Collectively, our findings reveal the molecular mechanism by which PKD intracellular localization and activity specifically regulate the surface localization of Kidins220 and raise the possibility of PKD1 and PKD2 interacting with PDZ proteins. This interaction may be crucial for the association of these isoforms with other proteins and the phosphorylation of other downstream targets, as well as for the regulation of protein cellular trafficking.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol 12,13-dibutyrate (PDBu), poly-l-lysine, and laminin were from Sigma. GF1 (GF 109203X or bisindolylmaleimide I) was purchased from Calbiochem. The polyclonal and monoclonal anti-laminin were from Sigma. GF1 (GF 109203X or bisindolylmaleimide I) was generously provided by Dr. S. Matthews (University of Dundee, UK) and anti-mouse secondary antibodies and [γ-$^{32}$P]ATP (370 MBq/ml) were from Amersham Biosciences; and Western Lightning™ chemiluminescence reagent plus was from PerkinElmer Life Sciences. Oligonucleotide primers were from Invitrogen. All other reagents were from standard suppliers or as indicated in the text.

**RNA Isolation and Reverse Transcription-PCR Analysis**—Total RNAs from PC12 cells, primary cortical neurons, or NB69 cells were isolated with TRizol reagent (Invitrogen) and then were reverse-transcribed into cDNAs. These cDNAs were PCR-amplified for 40 cycles using pairs of primers specific for rat PKD1, PKD2, and PKD3 (PKD1, sense at position 1106, 5′-GCC AAGGCG TTA AAT GTG AA-3′, antisense at position 1402, 5′-GGG GCT CGA GGT GTC GTA-3′; PKD2, sense at position 231, 5′-CTC GTA TCT CCA GTC TCT GC-3′, antisense at position 531, 5′-CAG AAC CAC CTC CAC CAA GT-3′; PKD3 sense at position 594, 5′-TGG TAA TGT CTA GGG TTA AA-3′, antisense at position 894, 5′-GTC GCC TCC TCT GAG TCA AA-3′) or human PKD1, PKD2, and PKD3 (PKD1/PKDmu sense at position 881, 5′-CGC ACA TCA TCT GCT GAA CT-3′, antisense at position 1181, 5′-CGT TCG GTG ACA ACC GTT TA-3′; PKD2, sense at position 1717, 5′-GGG CAG TTT GGA GTG GTG TA-3′, antisense at position 2018, 5′-ACC AGG ATC TGG GTG AG-3′; PKD3/PKDmu sense at position 1835, 5′-CCA GGA GGA ATA ACC TGA GA-3′, antisense at position 2134, 5′-TCT CTT TTT TTT CCC AGC TGT G-3′). After amplification, the primers yielded specific PCR fragments of 300 bp, whose identity was verified by sequencing using an Applied Biosystems automated DNA sequencer.

**cDNA Constructs and Site-directed Mutagenesis**—GFP-PKD1-WT and a kinase-deficient mutant GFP-PKD1-KD (with the mutation D733A) have been described previously (14, 17). GFP-PKD2-WT and GFP-PKD2-KD were kindly provided by Dr. T. Seufferlein (3). pcDNA4/TO-GFP-hPKD3-KD-WT and the kinase-deficient mutant pcDNA4/TO-GFP-hPKD3-KD-SIL and pcDNA4/TO-GFP-hPKD3-KD were kindly provided by Dr. T. Seufferlein (3). pcDNA4/TO-GFP-hPKD3-KD-SIL and pcDNA4/TO-GFP-PKD3/PKD3-KD-SVL mutants were obtained by substituting the three last amino acids, EDP, of hPKD3-KD sequence by PCR. The forward primers specific for rat PKD1, PKD2, and PKD3 (PKD1, sense at position 2134, 5′-GGC TCC TCT GAG TCA TCC AA-3′, near the polylinker region as external forward (5′-TAC CCA TGC CTG TTA CTC CTC AAG C-3′) was annealed at position 1589, close to the EcoRV restriction site located at position 1705), within the hPKD3 cDNA sequence. The reverse primers (annealing at position 2670) contained a new XbaI restriction site (5′-AGC GTT CTT CTC CTA CAG CTC AGT-3′ or to SVL (GAA GAT CCT to AGC ATT CTT, 5′-GCC GTA GAG CTC AGT GAT TAA AGA ATG CCT TC-3′) or to SVL (GAA GAT CCT to AGC ATT CTT, 5′-GCT CTA GAG CTC AGT GAT TAA AGA ACG CCT TC-3′). PCR fragments were digested with EcoRV/XbaI to substitute the same sequence in pcDNA4/TO-GFP-hPKD3-KD. The site-specific mutations within the PDZ-binding motif of PKD1, resulting in Ser$^{916}$ substitution, were generated by overlap PCR using pBluescript SK(−)-PKD1-KD as a template. Mutants were made by using an oligonucleotide annealing at position 2729, close to the Nhel restriction site located at position 2814, within the PKD1 cDNA sequence and a sequence downstream from the stop codon, corresponding to pBluescript SK(+), near the polylinker region as external forward (5′-TAC CCA
PDZ Ligands of PKD1/2 Control Kidins220 Traffic

CGA AAG CGA TGA CTC CAG-3') and reverse (5'-CCT CAC TAA AGG GAA CAA AAG CTG-3') primers, together with internal reverse and forward primers complementary to each other and containing the Ser916 substitutions. The sense primers containing the sequence changes encoding the desired mutations were as follows: PKD1-KD5916E (5'-TGA GGG TGT CGA GAT CCT CGT ATT-3') and PKD1-KD5916A (5'-TGA GGG TGT CGC CAT CCT CGT ATT-3'). After the second PCR, the amplified product, cut with Nhel/ Xba1, was used to replace the original Nhel/Xba1 segment in pEFBos-GFP-PKD1. The mutant GFP-PKD15916E was made by replacing the original Nhel/Xba1 segment of pEFBos-GFP-PKD1 with the same PCR product utilized to generate PKD1-KD5916E. To generate the mutant GFP-PKD1STOP, a new primer introducing a Stop codon (underlined) was used (5'-TGA GGG TGT CGA ATC CCT CGT ATT-3'). The mutant GFP-ΔCR-PKD1-KD was generated by replacing the Sphl-Xba1 fragment in the mutant GFP-ΔCR-PKD1 (described in Ref. 17) by the same fragment coming from the kinase-dead mutant PKD1-D733A (14). Constructs were sequenced using an Applied Biosystems automated DNA sequencer before they were used in transient expression experiments.

Cell Culture and Transfection—Primary dissociated E19 rat cortical primary cultures were prepared from cerebral cortices of 19-day-old fetal Wistar rats as described (18). Briefly, meninges were removed from the embryonic brains, and cortices were dissected. Tissue was resuspended in Neurobasal medium complemented with B-27 supplement (Invitrogen) and 2 mM glutamine. Cells were counted and seeded on laminin (4 μg/ml) and polylysine (10 μg/ml)-covered 35-mm dishes or glasses at a final concentration of 5 × 10⁵ and incubated at 37 °C in an atmosphere of 5% CO₂. Cells were used after 7 days in culture, as indicated in the text. PC12 rat pheochromocytoma cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 7.5% fetal calf serum, 7.5% horse serum, and 2 mM glutamine, in a humidified atmosphere containing 5% CO₂. When required, cells were treated with GF 1 (3.5 μM) for 1 h or PDBu (200 nM) for 2.5 h, as specified in the text. For transfection, PC12 cells and primary neurons were seeded at 50–60% confluence on laminin (4 μg/ml) and polylysine (10 μg/ml)-coated glass coverslips. Cells were transfected in serum-free medium by using 1 μg of DNA and 2.5 μl of Lipofectamine 2000® reagent (Invitrogen) per 35-mm dish, according to the manufacturer’s specifications, and 48 h later the cells were fixed and processed for immunofluorescence.

RNAi—Human NB69 neuroblastoma cells were transfected using Lipofectamine 2000 (Invitrogen) with pSuper or pSuper-PKD1-RNAi and pSuper-PKD2-RNAi vectors (kindly provided by Drs. A. Toker and P. Storz). Experiments were performed 72 h after transfection. For immunofluorescence analysis, pSuper vectors were cotransfected with pEFBos-GFP in a ratio of 10:1 in order to study Kidins220 localization in the green population of cells.

Immunoprecipitation and Western Blot Analysis—Transfected PC12 cells were lysed in radioimmunoprecipitation assay buffer (25 mM Tris- HCl, pH 7.6, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol) with protease and phosphatase inhibitors for 30 min at 4 °C, and lysates were then centrifuged for 10 min at 14,000 rpm. When needed, GFP-PKD1-KD was immunoprecipitated with anti-GFP antibodies. For Western blot analysis, total cell lysates or immunoprecipitates were analyzed by SDS-PAGE followed by transfer to nitrocellulose (Schleicher & Schuell). Membranes were blocked in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) plus 5% low-fat milk powder and incubated for 1 h at room temperature with the different primary antibodies in blocking solution. Membranes were incubated with the appropriate secondary antibodies conjugated to peroxidase as before, and immunoreactive bands were visualized by enhanced chemiluminescence.

In Vitro Kinase Assays—GFP-PKD1 and GFP-PKD1-KD autophosphorylation or trans-phosphorylation by recombinant active PKD1 were determined in GFP-PKD1 or GFP-PKD1-KD immunoprecipitates by the in vitro kinase assay as described previously (14). Briefly, immunoprecipitates were washed twice with radioimmunoprecipitation assay buffer and twice with kinase buffer (30 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 2 mM dithiothreitol) containing a 100 μM final concentration of [γ-³²P]ATP, in the absence or presence of purified active GST- PKD1 (100 ng), for 10 min at 30 °C. The reaction was stopped by adding 2X SDS-PAGE sample buffer (1 M Tris-HCl, pH 6.8, 6% SDS, 2 mM EDTA, pH 8.0, 4% 2-mercaptoethanol, 10% glycerol) and analyzed by SDS-PAGE and autoradiography or Western blotting with anti-PKD1-Ser(P)916-specific antibody. Peptide phosphorylation of Kidins220-Ser919 and Kidins220-Ala919 was determined by in vitro kinase assays as described previously (24).

Immunofluorescence and Confocal Microscopy—For immunofluorescence, transfected cells grown on coverslips were fixed for 2 min in 4% paraformaldehyde in phosphate-buffered saline at 4 °C, followed by methanol for 2 min at −20 °C. After blocking (1% bovine serum albumin in phosphate-buffered saline for 30 min), cells were incubated with the corresponding primary antibodies for 1 h at room temperature, and immunoreactivity was detected with the suitable fluorophore-conjugated secondary antibody before mounting on slides with Mowiol 4-88 (Harland Co., UK). All confocal images were acquired using an inverted Zeiss LSM510 Meta laser confocal microscope (Germany) with a 63× Plan-Apochromatic oil immersion objective and were normalized for each color separately. Confocal images presented are single sections of a series as specified in the text and figure legends. Images were processed for presentation with Zeiss LSM5 Image Browser and Adobe Photoshop 7.0 (Adobe Systems Inc.).

Quantitative Analysis—PC12 cells were transfected with the different PKD mutants. After immunostaining with Kidins220 antibody, 20–30 transfected cells were examined. Positive cells for GFP-PKD aggregates, Kidins220 aggregates, and aggregates where both proteins colocalized were counted (Figs. 1, 4, and 6). The percentage was calculated related to the total number of transfected cells examined.

RESULTS AND DISCUSSION

PKD1, PKD2, and PKD3 Differentially Regulate Cell Surface Localization of Kidins220 in Neurons and PC12 Cells—It has been published recently that the transport of basolateral membrane protein carriers in polarized epithelial cells is regulated by PKD1 and PKD2 but escapes from PKD3 control (22). These authors (22) suggest that the various PKD isoforms could recognize different vesicular cargoes as specific substrates and selectively control in this way their transport to the cell surface. Because Kidins220 was the first substrate identified for PKD1, as an integral membrane protein very abundant at the plasma membrane of neural cells (18, 24), we investigated whether PKD1, PKD2, and PKD3 could differentially regulate cell surface targeting of Kidins220 in E19 rat primary cortical neurons and PC12 cells. First, we analyzed the expression of the three isoforms in our cell systems by reverse transcription-PCR and discovered that primary cortical neurons expressed all members of the PKD family, whereas PC12 cells expressed only PKD1 and PKD2 (not shown). To study the possible differential effect of PKD isoforms on Kidins220 transport, we first expressed GFP-tagged wild
type or kinase-dead PKD1 (GFP-PKD1 and GFP-PKD1-KD, respectively) in both cell types. When expressing wild type PKD1, endogenous Kidins220 was present at the plasma membrane and in small punctate vesicles within the cell body (Fig. 1, A and B, upper panels), and GFP-PKD1 was found distributed throughout the cytosol, colocalizing at some membrane areas with Kidins220 staining (Fig. 1, A and B, upper panels). The immunostaining pattern of Kidins220 in these GFP-PKD1-transfected cells was very similar to the one observed in untransfected cells (data not shown and see Refs. 18 and 24), suggesting that the expression of wild type PKD1 did not significantly alter endogenous Kidins220 location. However, when GFP-PKD1-KD was expressed, Kidins220 was now mainly found accumulated in intracellular clusters, largely colocalizing with the inactive mutant (Fig. 1, A and B, lower panels). A quantitative analysis carried out in transfected PC12 cells (see “Experimental Procedures” for details) showed that GFP-PKD1-KD and Kidins220 colocalized in 80% of the intracellular aggregates (Fig. 1C, white bar). To explore further the decrease of Kidins220 staining at the surface of cells expressing the kinase-dead mutant GFP-PKD1-KD, transfected PC12 cells were also immunolabeled with Thy1. This glycosylphosphatidylinositol protein highly colocalizes with Kidins220, especially at the plasma membrane of PC12 cells in normal conditions (18). As shown in Fig. 1D, very little colocalization of Kidins220 and Thy1 staining could be observed at the surface of cells expressing GFP-PKD1-KD. From these results it can be concluded further that although Thy1 and Kidins220 are present at the same plasma membrane domains in neural cells, only Kidins220 surface targeting is controlled by PKD1 activity.

To study whether PKD1 was specifically regulating the trafficking of its substrate, we also examined the cellular distribution of the neotrophin receptors Trks and p75, because it has been reported that
Kidins220 forms a tripartite complex at the cell surface with these receptors (25, 31). In contrast to what was observed with Kidins220, the immunostaining of endogenous Trks, as well as p75, remained unaltered in both neurons and PC12 cells expressing GFP-PKD1-KD (Fig. 2, A and B) when compared with untransfected cells or cells transfected with GFP-PKD1 (not shown). These data show that PKD1 activity does not play a role in controlling the presence of the whole complex at the cell surface, further suggesting that it could be selectively regulating its substrate localization.

We next explored whether PKD2 and PKD3 activity could also govern the traffic of Kidins220. In order to do so, we transfected into neurons and PC12 cells kinase-deficient mutants of PKD2 (GFP-PKD2-KD) (3) and PKD3 (GFP-PKD3-KD) (30) and analyzed Kidins220 localization. When GFP-PKD2-KD was transfected into neuronal cells, it formed intracellular aggregates that colocalized with Kidins220 and showed the same pattern as that found in cells expressing the GFP-PKD1-KD mutant presented in Fig. 1 (not shown). Regarding PKD3, although GFP-PKD3-KD was retained intracellularly, Kidins220 immunostaining was normal in neural cells expressing this inactive mutant (Fig. 3, A and B, neurons and PC12 cells, respectively). Furthermore, the colocalization of Kidins220 with Thy1 at the plasma membrane of PC12 cells expressing GFP-PKD3-KD was preserved (Fig. 3C, see the purple color in the merge image). With these data we can conclude that PKD1 and PKD2 activities participate in the correct cell surface localization of Kidins220 and that PKD3 does not play a significant role in this process.

A Type I PDZ-binding Motif Identified in PKD1/2 Sequence Is Responsible for the Control of Kidins220 Surface Localization—In accordance to Yeaman et al. (22), a possible explanation for our results could be that PKD1, PKD2, and PKD3 have different substrate specificities and therefore can only control the transport of their specific substrates. However, such a fine selectivity seems unlikely considering the extremely high degree of homology shared by the catalytic domains of PKD1, PKD2, and PKD3 (5, 7). To study whether the three members of the PKD family could differentially phosphorylate Kidins220, we carried out peptide phosphorylation assays. We have published previously (24) that PKD1 phosphorylates Ser⁹¹⁹ within the Kidins220 sequence. By

![FIGURE 2. PKD1-KD does not affect the cell surface localization of two interacting partners of Kidins220, the neurotrophin receptors Trks and p75. Cortical primary neurons (A) and PC12 cells (B) were transfected with GFP-PKD1-KD and immunostained with anti-Trk (upper panels) and anti-p75 (lower panels) antibodies. Images show how the localization pattern of both proteins is not affected by the presence of kinase-dead mutant PKD1 (neither Trks nor p75 are accumulated with GFP-PKD1-KD in the intracellular compartment). Results are representative of three independent experiments. Confocal microscopy images correspond to single sections taken at the middle of the cells. A magnified detail of the merge images from neurons is depicted (merge zoom). Scale bar, neurons, merge images 10 μM, merge zoom images 5 μM; PC12 cells, merge images 10 μM.](image-url)
using a peptide containing Ser919 and a peptide in which this residue was substituted by an alanine, and by carrying out in vitro kinase assays, we demonstrated that PKD1, PKD2, and PKD3 were all able to efficiently phosphorylate Ser919 (not shown), suggesting that Kidins220 could be a good substrate for the three isoenzymes in vivo. This result indicates that substrate specificity might not be responsible for the differential control of Kidins220 transport. We then considered that PKD1, PKD2, and PKD3 could contain distinct features or undefined domains responsible for their differential effects on protein transport regulation and in particular on Kidins220 cell surface destination. When we compared the sequences of the three PKD isoforms, looking for outstanding differences, we found that PKD1 and PKD2 contain a potential type I PDZ-binding motif at the C terminus that is absent in PKD3 (Fig. 4A). The type I PDZ-binding sequences of PKD1 (Val–Ser–Ile–Leu), PKD2 (Ile–Ser–Val–Leu), and interestingly also of Kidins220 (Glu–Ser–Ile–Leu), are extremely similar, sharing the leucine and serine at the crucial positions 0 and −2, respectively, suggesting that the same PDZ proteins could form complexes with them. By contrast, PKD3 C terminus does not constitute a PDZ ligand of any type (Glu–Asp–Pro) (Fig. 4A). Because PDZ protein interactions can drastically influence the surface localization of multiple membrane proteins, the PDZ ligand of PKD1 and PKD2 could be responsible for the observed effects on Kidins220 distribution. To approach this issue, we investigated whether the PDZ-binding motif of PKD1 and PKD2 could change the isoform specificity of PKD3 and confer on it the ability to control Kidins220 localization. For this purpose, we first expressed in neural cells a GFP-PKD3-KD mutant where the last three C-terminal amino acids of PKD3 (EDP) had been substituted by the three amino acids corresponding to the PDZ-binding motif of PKD1 (SIL) (see scheme in Fig. 4A). In neurons and PC12 cells expressing this new mutant, Kidins220 staining was now enriched at the perinuclear region, where both proteins colocalized (Fig. 4, B and C, upper panels). Furthermore, a chimeric protein in which the most C-terminal sequence of PKD2 (SVL) replaced the equivalent region in GFP-PKD3-KD provoked the same effects on Kidins220 trafficking (Fig. 4, B and C, lower panels). A quantitative analysis carried out in PC12 cells transfected with GFP-PKD3-KD or GFP-PKD3-KD-SIL showed that although GFP-PKD3-KD hardly overlapped with the Kidins220 signal, the mutant carrying the last three amino acids of PKD1 colocalized in the same areas in 88% of the transfected population (Fig. 4D, white bar). Similarly to what was observed after expressing GFP-PKD1-KD, a significant decrease of Kidins220 staining at the cell surface of PC12 cells transfected with these two mutants (GFP-PKD3-KD-SIL and GFP-PKD3-KD-SVL) could be detected by double immunolabeling with Thy1 antibody (Fig. 4E). These results demonstrate that the consensus PDZ-binding sequence in PKD1 and PKD2, which may constitute an important docking site for the interaction of specific PDZ proteins, is critical for the specific control of Kidins220 traffic. On the other hand, these data suggest that some of the effects of PKD1, PKD2, and PKD3 on presumably so-called “specific cargoes” or substrates might not only be due to differences among isoform-specific kinase activities or substrates specificities but also to the binding partners (PDZ proteins for PKD1 and PKD2 and maybe other adaptors for PKD3) recruited through their unique C terminus.

Kidins220 Colocalizes with PKD1-KD and PKD3-KD-SIL at the TGN of Neural Cells—So far our results show that Kidins220 localization at the plasma membrane is highly altered in neural cells expressing GFP-PKD1-KD, GFP-PKD2-KD, and GFP-PKD3-KD-SIL or GFP-PKD3-
Because the kinase-dead mutants of the different PKD isoforms are mainly bound to the TGN in cells of epithelial origin, and their expression causes the inhibition of the transfer of cargo from the TGN to the plasma membrane (9, 10, 12, 19, 21–23), we next examined whether our mutants were bound to the TGN and whether they could be retaining Kidins220 at this compartment in neural cells. PC12 cells transfected with GFP-PKD1-KD, GFP-PKD3-KD, and GFP-PKD3-KD-SIL were double-stained with antibodies against Kidins220 and the TGN marker TGN38. We observed that a significant portion of Kidins220 colocalized with GFP-PKD1-KD and GFP-PKD3-KD-SIL, especially at the perinuclear area, that was also labeled with TGN38 (Fig. 5, upper and lower panels). Although both GFP-PKD3-KD and GFP-PKD3-KD-SIL localized to some extent with the TGN marker, we also noted that Kidins220 was only found there in cells expressing the PKD3 inactive mutant containing the PDZ-binding motif of PKD1 (Fig. 5, compare middle and lower panels). Our results show that all three PKD kinase-dead mutants can be bound to TGN membranes also in neural cells and that the retention of Kidins220 in this compartment depends on the presence of the PDZ-binding motif on PKD molecules. These data also suggest that inactive PKD molecules bearing a PDZ ligand could be blocking the exit of vesicular exocytic carriers containing Kidins220 from the TGN in our cell system.

**The Phosphorylation of Ser916 within the PDZ-binding Motif PKD1 Is Critical for the Regulation of Kidins220 Transport**—PKD1 autophosphorylates at Ser916, and the PKD1-KD mutant is unable to undergo autophosphorylation on this residue (16). Because Ser916 is the critical residue at position −2 of the PDZ-binding motif of PKD1, this opens the perspective for the control of PDZ protein interactions with this domain by phosphorylation. Typical class I PDZ ligands contain a serine or threonine at position −2, and modulation of PDZ interactions by phosphorylation of this residue by different protein kinases controlling surface targeting has been clearly shown for many membrane and PDZ proteins.
PKD1 trans-phosphorylates Ser\textsuperscript{916} within PKD1-KD in vitro—To provide further evidence supporting that PKD1 could trans-phosphorylate other PKD1 molecules, total lysates from PC12 cells transiently transfected with GFP-PKD1 or GFP-PKD1-KD were immunoprecipitated with anti-GFP antibodies, and an in vitro kinase assay was carried out in the absence or presence of purified recombinant active PKD1 (Fig. 8). Compared with GFP-PKD1 (Fig. 8, 2nd lane), GFP-PKD1-KD alone (Fig. 8, 4th lane) did not show either kinase activity

Because Ser\textsuperscript{916} is an autophosphorylation site on PKD1, and because trans-phosphorylation among PKD1 molecules has never been reported, it is tempting to speculate, considering the present data, that activation of endogenous PKD1 could be trans-phosphorylating this residue on PKD1-KD and rescuing in this way Kidins220 transport. In an effort to check this hypothesis, a kinase-dead mutant unable to suffer phosphorylation at Ser\textsuperscript{916} (GFP-PKD1-KD\textsuperscript{916A}) was now transfected. Most strikingly, GFP-PKD1-KD\textsuperscript{916A} behaved as a constitutive repressor of Kidins220 transport to the plasma membrane, even in the presence of PDBu (Fig. 7B), showing that endogenous PKD1 might need Ser\textsuperscript{916} intact and in a phosphorylatable state within the kinase-dead mutant in order to restore Kidins220 traffic. We then tested if Ser\textsuperscript{916} was indeed phosphorylated in GFP-PKD1-KD by Western blot analysis with a phospho-specific antibody (anti-PKD-Ser(P)\textsuperscript{916}) of total lysates from PC12 cells transfected with the mutant and treated with GF I and/or PDBu (Fig. 7C). After incubation with phorbol esters, GFP-PKD1-KD appeared phosphorylated at Ser\textsuperscript{916} (Fig. 7C, 2nd lane), and this phosphorylation was prevented by pretreating the cells for 1 h with GF I (Fig. 7C, 4th lane). These observations provided new evidence supporting the importance of Ser\textsuperscript{916} phosphorylation on the regulation of Kidins220 sorting controlled by PKD1, and strongly suggested that in vivo the endogenous PKD1 could be trans-phosphorylating GFP-PKD1-KD at Ser\textsuperscript{916}.
Recombinant active PKD1 was able to phosphorylate the kinase-dead mutant in vitro (where it appeared radioactive and phosphorylated at Ser916) and also the wild type protein very slightly (Fig. 8, 3rd and 1st lanes, respectively), demonstrating that trans-phosphorylation between PKD1 molecules occurs. These findings lead to several important considerations. For many years, the phosphorylation of Ser916 has been taken as a direct and easy way to measure PKD1 activity with phospho-specific antibodies, allowing the scientific community to avoid radioactive in vitro kinase assays. The use of the phospho-antibody to assess the activation state of physiological endogenous PKD1 would still be appropriate. However, our data question the use of these antibodies to determine PKD1 activity in certain experiments, especially in those aimed to show the activation state of different mutants or constructs in cells where endogenous levels and activity of the enzyme are high. On the other hand, although most likely in vivo autophosphorylation would take place with higher efficiency than trans-phosphorylation, the results obtained here also suggest that the effects of kinase-inactive mutants can be dramatically different depending primarily on the activation state of endogenous PKD1.

PKD1-KD Association to Intracellular Membranes Is Crucial to Control the Transport of Kidins220—So far our results show that PKD1 activity controls the localization of Kidins220 at the cell surface by a mechanism that requires the phosphorylation of Ser916 within its PDZ-binding motif. We now wanted to explore whether PKD1 intracellular localization could be also important for the regulation of this process. As mentioned previously, PKD1-KD is constitutively bound to TGN membranes through the CR domain (9, 10, 12, 21). We generated a PKD1-KD mutant in which the CR domain had been deleted (H9004 CR-PKD1-KD, Fig. 9A and B) and studied whether it could still control Kidins220 transport. GFP-H9004 CR-PKD1-KD transfected into PC12 cells was not present in the intracellular clusters where GFP-PKD1-KD is typically found and showed a cytosolic localization (Fig. 9B). More importantly, in cells expressing this new mutant the distribution of Kidins220 was normal (Fig. 9B). This last result shows
that the association of PKD1-KD to intracellular membranes at the TGN is a prerequisite to control Kidins220 traffic. All together, our data strongly support a molecular mechanism by which PKDs control Kidins220 transport to the cell surface by involving at least two steps as follows: first, the association of PKD to intracellular TGN membranes, and second, the auto/trans-phosphorylation of the PDZ-binding motif of the kinase at Ser916 controlling its interaction with PDZ proteins in this compartment.

Concluding Remarks—Functional studies of the different PKD isoforms have shown isoform specificity on the control of nonpolarized and polarized protein transport and fission of exocytic carriers from the TGN to the cell surface (22, 23), but the molecular mechanism involved in the possible selective regulation of the traffic of their specific substrates remained obscure. Here we have identified the C-terminal end of the various PKD isoforms, which constitutes a PDZ-binding motif in PKD1 and PKD2 but not in PKD3, and the phosphorylation of Ser916 contained therein in PKD1 to be responsible for the differential control of Kidins220 plasma membrane localization in neural cells. PKD3 would not be controlling Kidins220 traffic because it does not contain this motif at its C-terminus.

Depending on the cell context and the stimulation conditions, the different PKD isoforms can be targeted to different intracellular locations such as the Golgi apparatus, the cytosol, the plasma membrane, or the nucleus (5, 7). Most of the effects of PKD controlling protein transport have been described in cells where PKD1 (endogenous or ectopically expressed) is bound to the TGN and have been studied by using kinase-inactive mutants (9, 10, 12, 19–22). It has been also described, at least for PKD1, that the inactivation of the kinase renders a protein that is constitutively bound to the TGN through the CR domain (9, 10, 12, 21). Our experiments show that in neural cells under basal conditions endogenous PKD1/2 (18) and ectopically expressed wild type isoforms are mainly cytosolic, but the kinase-defective mutants of the three PKD isoforms localize to intracellular membranes enriched in the TGN, a phenotype similar to the one observed in nonpolarized or polarized epithelial cells (22). Here we also demonstrate that the association of PKD1 kinase-inactive mutants to intracellular membranes is critical for the control of Kidins220 transport (Fig. 9). Altogether, these data further support the idea that PKD intracellular localization and association to the TGN might be the first crucial step in mediating the regulation of protein transport at this compartment. Our results also strengthen the fact that the distinct cellular compartmentalization of PKDs in different cells, tissues, developmental stages, or pathological circumstances may lead to dramatically different consequences on cellular functions such as protein transport.

With our results we propose a model providing a possible molecular mechanism by which TGN-bound PKD1 can regulate the exit of
Kidins220 from this compartment through an autophosphorylation-regulated interaction of its PDZ-binding motif with a putative PDZ protein (Fig. 10). This model would also operate for PKD2 but not for PKD3. According to our model, the cytosolic enzyme would not be able to control protein transport. In cell types or under circumstances in which PKD is not bound to the TGN, it would not be regulating this process. In our cell systems under the studied conditions, ectopically expressed wild type and endogenous PKDs are mainly cytosolic, not bound to the TGN (see Fig. 1A) (18). In this context, and in agreement with our hypothesis, in neural cells the overexpression of wild type PKD1/2 and, more importantly, the RNAi of endogenous PKD1/2 (not shown) are not having any significant influence on Kidins220 cellular distribution. To exert a control on Kidins220 traffic, PKD needs to be associated with the TGN. Supporting this notion, the deletion of the CR domain responsible for DAG binding and association to membranes has been deleted (A). PKD cells were transfected with GFP-PKD1-KD (upper panels) or GFP-ΔCR-PKD1-KD (lower panels) mutants and Kidins220 immunostaining was analyzed (B). GFP-ΔCR-PKD1-KD presents a cytosolic localization and is not present in intracellular clusters. Most importantly, this new mutant is not able to control Kidins220 traffic, suggesting that the association of PKD1-KD to intracellular membranes at the TGN is crucial for the regulation of Kidins220 transport. Results are representative of three independent experiments. Conical microscopy images correspond to single sections taken at the middle of the cells. Scale bar, 10 μm.

FIGURE 9. A kinase-dead mutant of PKD1 lacking the CR domain does not alter Kidins220 localization. Scheme of PKD1-KD and ΔCR-PKD1-KD shows a mutant in which the CR domain responsible for DAG binding and association to membranes has been deleted (A), PKD cells were transfected with GFP-PKD1-KD (upper panels) or GFP-ΔCR-PKD1-KD (lower panels) mutants and Kidins220 immunostaining was analyzed (B). GFP-ΔCR-PKD1-KD presents a cytosolic localization and is not present in intracellular clusters. Most importantly, this new mutant is not able to control Kidins220 traffic, suggesting that the association of PKD1-KD to intracellular membranes at the TGN is crucial for the regulation of Kidins220 transport. Results are representative of three independent experiments. Conical microscopy images correspond to single sections taken at the middle of the cells. Scale bar, 10 μm.

FIGURE 8. Trans-phosphorylation among PKD1 molecules. Total lysates from PC12 cells transiently transfected with GFP-PKD1 or GFP-PKD1-KD were immunoprecipitated (IP) with anti-GFP antibodies. Immunoprecipitates were subjected to an in vitro kinase assay in the absence (∼) or presence (+) of recombinant purified active PKD1. Note that the mutant alone (4th lane) presents neither kinase activity (autoradiograph, upper panel) nor phosphorylation on Ser916 (Western blot (WB), middle panel) compared with wild type kinase (2nd lane). However, in the presence of active PKD1, the kinase-dead mutant is phosphorylated at Ser916, demonstrating that trans-phosphorylation occurs among PKD1 molecules. GFP-PKD1 and GFP-PKD1-KD levels in the immunoprecipitates were determined by Western blotting with anti-GFP antibodies (lower panel). Results are representative of three independent experiments.

The simplest model that will bring together PKD1, Kidins220 (or other cargoes bearing a certain PDZ ligand), and PDZ proteins would be one where PKD1 and Kidins220 are ligands of the same multi-PDZ domain protein under circumstances where no phosphorylation of the PDZ-binding motif of PKD1 occurs (e.g. resting conditions or expression of PKD1-KD). The unphosphorylated C terminus of PKD1 is a bona fide PDZ-binding motif that might constitute a high affinity docking site for the specific PDZ protein. The formation of these PDZ complexes may strictly depend on PKD localization at the TGN. The kinase-dead mutants constitute the most extreme example of TGN-bound PKDs. Because PKD1 kinase-inactive mutant is trapped in the TGN and it cannot autophosphorylate, the correspondingly bound PDZ protein would be retained there, blocking the fission of TGN exocytic carriers containing Kidins220 from the TGN through the interaction with this family of proteins. This model would easily explain why the expression of inactive PKD mutants blocks the traffic of specific TGN-derived vesicle carriers described by others (22) in an isoform-specific manner and how PKD activity can normalize the transport process.

Kidins220 from this compartment through an autophosphorylation-regulated interaction of its PDZ-binding motif with a putative PDZ protein (Fig. 10). This model would also operate for PKD2 but not for PKD3. According to our model, the cytosolic enzyme would not be able to control protein transport. In cell types or under circumstances in which PKD is not bound to the TGN, it would not be regulating this process. In our cell systems under the studied conditions, ectopically expressed wild type and endogenous PKDs are mainly cytosolic, not bound to the TGN (see Fig. 1A) (18). In this context, and in agreement with our hypothesis, in neural cells the overexpression of wild type PKD1/2 and, more importantly, the RNAi of endogenous PKD1/2 (not shown) are not having any significant influence on Kidins220 cellular distribution. To exert a control on Kidins220 traffic, PKD needs to be associated with the TGN. Supporting this notion, the deletion of the CR domain responsible for DAG binding and association to membranes has been deleted (A). PKD cells were transfected with GFP-PKD1-KD (upper panels) or GFP-ΔCR-PKD1-KD (lower panels) mutants and Kidins220 immunostaining was analyzed (B). GFP-ΔCR-PKD1-KD presents a cytosolic localization and is not present in intracellular clusters. Most importantly, this new mutant is not able to control Kidins220 traffic, suggesting that the association of PKD1-KD to intracellular membranes at the TGN is crucial for the regulation of Kidins220 transport. Results are representative of three independent experiments. Conical microscopy images correspond to single sections taken at the middle of the cells. Scale bar, 10 μm.

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Although PKD1-KD-Ser^916 will be still retained at the TGN, the transport of specific ligands of this PDZ protein (i.e. Kidins220) to the cell surface would be now normalized. This model would also operate in certain cell types and situations in which endogenous or wild type PKD1 is bound to the TGN.

The model we propose here is further supported by the fact that some PDZ proteins interact with molecular motors bound to the actin cytoskeleton, such as kinesin and myosin (37–39). Transport of many membrane receptors, transporters, and ion channels from the TGN to specific sites of the cell surface depends on motor proteins (40, 41), and PDZ proteins have been suggested as attractive candidates that could serve as adapters mediating the specificity of interactions between membranous cargo proteins and cytoskeletal motors (42). It is tempting to speculate that PDZ proteins and the pulling forces of the cytoskeletal molecular motors could participate on the fission of vesicles carrying their ligands from the TGN, and that PKD activity is controlling this step through the phosphorylation of its PDZ-binding motif.

Our model could constitute a general mechanism for the regulation of the sorting of other cargoes by PKD1/2 activity by modulating the release of PDZ proteins from its auto/trans-phosphorylated PDZ-binding motif. Determining which is/are the PDZ protein(s) responsible for the dramatic effects exerted by the different PKD isoforms on the transport of Kidins220 or other specific cargoes will undoubtedly provide a better understanding of how protein trafficking is controlled in the complex membrane/cytoskeletal networks.

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PDZ Ligands of PKD1/2 Control Kidins220 Traffic

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