PKD1, PKD2, and Their Substrate Kidins220 Regulate Neurotensin Secretion in the BON Human Endocrine Cell Line*

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Neurotensin (NT) is a gut peptide that plays an important role in gastrointestinal secretion, motility, and growth as well as the proliferation of NT receptor-positive cancers. Protein kinase D (PKD) family members (PKD1, 2, and 3) have been identified as important regulators of secretory transport at the trans-Golgi network. Previously, we showed that PKD1 contributes to stimulated NT secretion; however, the mechanisms are not entirely clear. Here, we show that Kidins220, which is a substrate of PKD proteins in neuroendocrine cells, is localized in the ends of the processes of BON cells, similar to the expression pattern of NT vesicles, and translocates to the membrane and large vesicle-like structures formed in response to phorbol 12-myristate 13-acetate treatment. The short hairpin RNA targeting Kidins220 inhibits NT secretion in parental BON cells or BON cells stably expressing the gastrin-releasing peptide receptor treated with either phorbol 12-myristate 13-acetate or bombesin, respectively. Furthermore, we demonstrate that endogenous PKD1, PKD2, and Kidins220 co-exist with NT-containing vesicles. Overexpression of the kinase-dead PKD1 abrogates Kidins220 expression and NT vesicle formation. Our data establish a physiological link between the PKD/Kidins220 pathway and NT-containing vesicles and suggest the role of this pathway in the regulation of hormone secretion. Because NT is an important gut hormone that affects secretion, inflammation, and both normal and tumor cell growth, our findings identify a novel signaling pathway that may be amenable to drug targeting for clinical applications.

The peptide neurotensin (NT),2 identified and characterized by Carraway and Leeman (1) from bovine hypothalamus, is produced in the brain and in enteroendocrine cells localized predominantly in the jejunum and ileum. The gastrointestinal effects of NT include facilitation of fatty acid translocation from the intestinal lumen, regulation of gut motility, and stimulation of the growth of normal gut mucosa as well as other tissues such as the adrenal gland, hepatocytes, and fibroblasts (2, 3). NT also stimulates growth of certain pancreatic, colonic, and prostatic cancers bearing NT receptors (2, 3), and recently a novel role of NT as a proinflammatory peptide has been demonstrated in the gut (4, 5). Together, these studies identify NT as an important intestinal hormone in normal physiologic functions and, furthermore, as a potential mediator of pathologic effects such as tumor growth and inflammation.

Previously, we established and characterized a novel endocrine cell line, BON, derived from a human pancreatic carcinoma (6). BON cells synthesize and secrete NT peptide and process the NT peptide in a manner analogous to that of enteroendocrine cells in the small bowel, thus serving as a useful model for enteroendocrine cell secretion (6). Also, BON cells have been instrumental in delineating signaling molecules that contribute to the stimulated secretion of hormones and amines from carcinoid tumors (7). Focusing on the intracellular signaling pathways that regulate NT release from BON cells, we found that protein kinase C, especially the isoforms α and δ and the protein kinase C substrate MARCKS (myristoylated alanine-rich C kinase substrate), contributes to stimulated NT secretion (8, 9). We further demonstrated that protein kinase D (PKD), a novel serine/threonine kinase family, also plays a role downstream of protein kinase C in the regulation of NT release (10).

PKD has been implicated as an important regulator of the transport from trans-Golgi network (TGN) to the plasma membrane (11–13). The PKD family consists of three members, PKD1, PKD2, and PKD3 (14). PKD1 is recruited to the TGN through interaction with diacylglycerol and is subsequently activated by phosphorylation to promote fission of carriers that deliver cargo to the plasma membrane (15). Similar to PKD1, PKD2 can be activated by phorbol esters, phospholipids, and the cholecystokinin B/gastrin receptor (16). PKD2 and PKD3 also affect the TGN-to-plasma membrane trafficking in non-polarized HeLa cells that only express endogenous PKD2 and 3, but not PKD1 (11). Using a polarized model of Madin-Darby canine kidney cells that express all three PKD isoforms, it was found that PKD1 and 2, but not 3, participate in the trafficking from TGN to the
basolateral membrane (11). In recent studies, downstream substrates of PKD have been identified. Kinase D-interacting substrate of 220 kDa (Kidins220) was first identified as a PKD physiological substrate in PC12 cells (17). Kidins220 is an integral plasma membrane protein predominantly expressed in brain and neurons. PKD1 and PKD2, but not PKD3, specifically regulate the surface localization of Kidins220 in PC12 cells.

In this study, we extended our findings regarding the important roles of PKD proteins and determined the mechanisms by which PKD proteins regulate NT secretion. Here, we demonstrate that PKD2 and the PKD protein substrate Kidins220 regulate stimulated NT secretion. PKD1, PKD2, and Kidins220 display the same localization pattern as NT vesicles at the ends of the processes of BON cells and co-exist with NT vesicles. The findings in our current study provide the first evidence to suggest the regulation of NT hormone secretion through the PKD/Kidins220 signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—The GST-tagged PKD plasmids, including wild-type PKD1 and 2, were provided by Dr. Vivek Malhotra (University of California, San Diego) (11). Phorbol 12-myristate 13-acetate (PMA), bombesin (BBS), and thapsigargin were purchased from Sigma. The anti-PKD1 and NT antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PK2D, calnexin, protein disulfide isomerase, Kidins220, phospho-Kidins220 (S919), and CCAAT/enhancer-binding protein homologous protein (CHOP) antibodies were from Abcam, Inc. (Cambridge, MA). The anti-phospho-PKD2 (Ser876) was from Upstate (Lake Placid, NY). The anti-TGN46 antibody was from Serotec (Raleigh, NC). The GST-fluorescein isothiocyanate antibody for immunostaining was from Alpha Diagnostic (San Antonio, TX). Alexa Fluor secondary antibodies for fluorescent staining were from Invitrogen. The secondary antibodies for Western blotting were from Pierce. The anti-GST antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PKD1 and NT antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PKD1, PKD2, calnexin, protein disulfide isomerase, Kidins220, phospho-Kidins220 (S919), and CCAAT/enhancer-binding protein homologous protein (CHOP) antibodies were from Abcam, Inc. (Cambridge, MA). The anti-phospho-PKD2 (Ser876) was from Upstate (Lake Placid, NY). The anti-TGN46 antibody was from Serotec (Raleigh, NC). The GST-fluorescein isothiocyanate antibody for immunostaining was from Alpha Diagnostic (San Antonio, TX). Alexa Fluor secondary antibodies for fluorescent staining were from Invitrogen. The secondary antibodies for Western blotting were from Pierce. The anti-GST antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture, Transfection, and Stable Cell Lines**—The BON cells were established by cell sorting and maintained in RPMI-G418 (500 μg/ml). Transient transfections were performed using Lipofectamine 2000 from Invitrogen.

**Protein Preparation and Western Blotting**—Protein preparations and Western blotting were performed as described previously (9). In brief, the cells were lysed with cell lysis buffer from Cell Signaling Technology, Inc. (Danvers, MA). Equal amounts of protein were resolved on NuPAGE BisTris gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes; the membranes were incubated with primary antibodies overnight at 4 °C followed by secondary antibodies conjugated with horseradish peroxidase. Membranes were developed using the ECL detection system.

**Immunofluorescent Staining and Confocal Microscopy**—BON cells were grown on glass coverslips in 24-well plates. 2 or 3 days after seeding or transfection, cells were treated with vehicle (Me2SO) or PMA (100 nM) for 30 min. After treatment, cells were fixed with 4% paraformaldehyde for 20 min at 37 °C. After three washes with PBS, the cells were permeabilized with 0.3% Triton X-100 for 15 min at 37 °C and blocked with 1% bovine serum albumin/PBS for 10 min. The cells were incubated with the primary antibody diluted with 1% bovine serum albumin/PBS for 2 h at room temperature. Cells were washed three times with PBS and incubated with Alexa-conjugated secondary antibody diluted by 1:500 in 1% bovine serum albumin/PBS. The fluorescence staining was observed under an LSM 510 META confocal system configured with an Axiovert 200M inverted microscope (Zeiss, Jena, Germany). Images were acquired using a plan-apochromat ×63, 1.4-NA oil immersion objectives, and the 488- or 568-nm line of an argon-ion laser for excitation. The image acquisition and processing were carried out using the Zeiss LSM510 work station (version 3.0) and the Zeiss Image Browser (version 3.1) software.

**One-step Real-time RT-PCR**—Total RNA was extracted from BON cells and real-time RT-PCR performed using Applied Biosystems assays-on-demand 20X assay mix of primers and TaqMan MGB probes (FAMTM dye-labeled) for target genes PRKD1 (ID Hs00177037_m1), PRKD2 (ID Hs00212828_m1), and PRKD3 (Hs00178657_m1) and predeveloped 18 S rRNA(VICTM dye-labeled probe) TaqMan® assay reagent (P/N 4319413E) for endogenous control. Separate tubes (singleplex) one-step RT-PCR was performed with 80 ng of RNA for both target gene and endogenous control. The reagent was TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR were: reverse transcription 48 °C for 30 min, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (repeat 40 times) on ABI7000. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T (ΔΔC_T) method as described by the manufacturer (Applied Biosystems). The amount of target (2^-ΔΔC_T) was obtained by normalizing to the endogenous reference (18 S) and relative to a calibrator (one of the experimental samples). All reactions were performed in triplicate.
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FIGURE 1. Kinase-dead PKD decreases NT secretion. A, total RNA was extracted from BON cells, and real-time RT-PCR was performed using probes for the PKD1, PKD2, and PKD3 genes (i.e. PRKD1, PRKD2, and PRKD3, respectively) and analyzed as described under “Experimental Procedures.” Experiments were performed at least three times. B, BON cells were transiently transfected with the kinase-dead PKD2 as well as PKD1 for 24 h and treated with PMA and NT secretion measured (top). (*, p < 0.05 versus control empty vector; †, p < 0.05 versus control empty vector plus PMA; n = 6). Cells were lysed and Western blotting analysis performed with anti-GST antibody (bottom). Experiments were performed at least three times. C, left panel, BON/GFP-GRPR cells were treated with BBS (0, 1, 10, and 100 nM) for 1 h, and NT secretion was measured (right panel). Parental BON cells were treated with BBS or PMA and NT secretion measured. (*, p < 0.05 versus control, n = 6). Experiments were performed at least three times. D, BON/GFP-GRPR cells were transiently transfected with the kinase-dead PKD1 and PKD2 for 24 h and treated with BBS (10 nM) and NT secretion measured (top). (*, p < 0.05 versus control empty vector; †, p < 0.05 versus control empty vector plus BBS; n = 6). Cells were lysed and Western blotting analysis performed with anti-GST antibody; β-actin was probed as a loading control (bottom). Experiments were performed at least three times.

Generation of Kidins220 Short Hairpin RNA (shRNA) Vectors—The BLOCK-iT Inducible H1 RNAi Entry Vector kit was purchased from Invitrogen. The single-stranded DNA oligos were designed using Invitrogen’s RNAi Designer on-line. Two target sequences were selected to knock down Kidins220: Sh1-top, caccgccagagacagcatatggatcatgataaattcgtcttg; Sh1-bottom, aaaaagccagagagcagatcagtattttcgaatactgatctgctctctggc; Sh2-top, cacccgatatactcgcagagcatctctttgtgctgctgattc; Sh2-bottom, aaagatagtaacctctccgagacagatctctttgtgctgctgattc.

Double-Stranded oligos were generated and cloned into the pENTR/H1/TO vector following the manufacturer’s instructions. The LacZ 2.1 control vector was generated using the LacZ2.1 double-stranded oligos from the kit. The positive clones for both Kidins220 and LacZ were screened by sequencing to confirm the sequence of the double-stranded oligo insert.

Statistical Analysis—Because of heterogeneous variability among treatment groups, data were transformed using logarithm to the base 10 for data analysis. The log-transformed data were analyzed using analysis of variance for a two-factor experiment (Fig. 1, B and D, and Fig. 6, B and C) or a one-factor experiment (Fig. 1C). The two factors were vector and treatment. The one factor was BBS dose (0, 1, 10, and 100 nM) or PMA. Main effects and interactions were assessed at the 0.05 level of significance. Multiple comparisons were conducted using a t statistic with the standard error computed from the residual mean square in the analysis of variance and the comparisonwise error rate with Bonferroni adjustment for the number of comparisons. Statistical computations were carried out using PROC GLM in SAS®, Release9.1 (19).

RESULTS

PKD2 Regulates PMA-stimulated NT Secretion in BON Cells—We have previously demonstrated a role for PKD1 in stimulated NT secretion (10). Because PKD2 and PKD3 are known to play more important roles in protein transport from Golgi to the cell surface, we determined whether BON cells express these two PKD isoforms. Using real-time RT-PCR, we found that PKD1 and PKD2 are abundantly expressed; PKD3 mRNA was detectable but at very low levels in BON cells (Fig. 1A). Based on the abundant expression of PKD2 in BON cells, we further determined whether PKD2 plays a role in stimulated NT secretion. We performed transient transfections of BON cells with the
PKD1 and PKD2 Exist in the Ends of the Processes of BON Cells—PKD1, PKD2, and PKD3 are distributed primarily in the cytosol, Golgi, or nucleus; activated PKD proteins can shuttle between different compartments (12, 14). To investigate the mechanisms for the PKD1 and PKD2 regulation of PMA-stimulated NT secretion in BON cells, we next analyzed the intracellular localization of endogenous PKD1 and PKD2 in resting or activated BON cells by immunostaining and confocal microscopy. Previously, we have shown that PKD1 was expressed in the cytosol as well as in the nucleus and translocated from the cytosol to the membrane upon PMA stimulation (10). In our current study, we found that the endogenous PKD1 (Fig. 2, left panel, top) and PKD2 (middle panel, top) also localized to the ends of the processes of unstimulated BON cells. This expression pattern is very similar to the location of NT-containing vesicles in BON cells (right panel, top). Discrete membrane-localized PKD1 (left panel, bottom) and PKD2 (middle panel, bottom) was noted upon PMA treatment. NT staining displayed a pattern similar to PKD1 and PKD2 in the presence of PMA (right panel, bottom). In addition, punctate structures were noted in the cytosol with all PKD1, PKD2, and NT staining. Taken together, the localization of endogenous PKD1 and PKD2 at the ends of the processes where NT vesicles are localized demonstrates the close relationship between PKD1 and PKD2 with NT vesicle release. PKD1 and PKD2 may participate in the formation of vesicles from the TGN and, therefore, regulate NT secretion.

PKD1 and PKD2 Preferentially Colocalize with NT Vesicles but Not TGN or ER—The immunostaining pattern of PKD1 and PKD2 in the ends of the processes of BON cells suggested the possibility that PKD1 and PKD2 might colocalize with NT vesicles. We next performed double immunofluorescence staining and confocal microscopic analysis using anti-PKD1 or -PKD2 antibodies and anti-NT antibody to detect the colocalization of endogenous PKD1 or PKD2 with endogenous NT vesicles. Both PKD1 (Fig. 3A) and PKD2 (Fig. 3B) partially colocalized with the NT-containing vesicles. To further confirm this expression pattern, we established BON cell lines expressing GFP-tagged NT (BON/GFP-NT), and immunostaining with anti-PKD1 antibody was performed. Images taken by confocal microscopy further demonstrate the colocalization of PKD1 and NT vesicles (Fig. 3C).

It has been reported that PKD is partially localized with the Golgi compartments in a human hepatocellular carcinoma cell line and in human antral gastrin-secreting cells (13, 22). To test whether this occurs in BON cells, we utilized the TGN46 antibody as a marker of TGN to perform double immunofluorescence staining and confocal microscopic analysis. The localization of both PKD1 and PKD2 in TGN was not noted in either untreated or PMA-treated cells (data not shown). These results demonstrate that the vesicles do not contain Golgi components. To determine whether PKD1 or PKD2 colocalizes with ER, we performed immunostaining using anti-calnexin or protein disulfide isomerase antibodies as ER markers. PKD1 and PKD2 did not colocalize with ER in either the presence or absence of PMA (data not shown). Taken together, our data show that both PKD1 and PKD2 are preferentially colocalized with the NT vesicles, but not with the TGN or ER, suggesting PKD1 and PKD2 may regulate NT vesicle formation and are transported along with NT vesicles to the ends of the processes.

Kidins220 Is Expressed in BON Cells and Partially Colocalizes with NT Vesicles—Kidins220 was first found to be a substrate for PKD proteins (17). It has been demon-
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FIGURE 4. Kidins220 partially colocalizes with NT vesicles. A, BON cells were plated on coverslips in 24-well plates and grown for 48 h. Cells were treated without (a) or with PMA (b), forskolin (FSK) (c), or PGE2 (d). Immunostaining was performed with anti-Kidins220 antibody. Experiments were performed at least three times. B, BON/GFP-NT cells were plated on coverslips in 24-well plates and grown for 48 h. Immunostaining with anti-Kidins220 antibody and confocal microscopic analysis were performed. Experiments were performed at least three times.

strated that Kidins220 is an integral membrane protein selectively expressed in brain and neuroendocrine cells such as PC12 cells (17). BON is an endocrine cell line; therefore we were interested to determine whether Kidins220 is expressed in BON cells. As shown in Fig. 4A, Kidins220 exists in the ends of the processes in untreated BON cells, which is similar to the PKD1, PKD2 and NT expression patterns (Fig. 4A, panel a). Upon PMA treatment, the expression of Kidins220 at the ends of the processes disappeared (panel b), and instead, expression on the plasma membrane was noted. Surprisingly, PMA treatment caused formation of vesicle-like structures that are Kidins220-positive and very similar to the expression pattern of PKD1 and PKD2 upon PMA treatment (panel b). Forskolin (FSK) (panel c) and PGE2 (panel d), both of which stimulate NT secretion via the cAMP/protein kinase A pathway (18), were utilized as controls. In contrast to PMA, forskolin and PGE2 failed to alter the expression pattern of Kidins220, suggesting the specific response of Kidins220 to protein kinase C pathway activation.

We next analyzed the colocalization of endogenous Kidins220 with NT vesicles in the BON/GFP-NT cells using an anti–Kidins220 antibody (Fig. 4B). Consistent with the endogenous NT vesicles, GFP-tagged NT displayed a similar expression pattern (Fig. 4B, left panel). Also, the staining of Kidins220 showed a similar expression pattern as the NT vesicles (middle panel). The merged images demonstrate the partial colocalization of Kidins220 with the NT-containing vesicles (right panel). Taken together, these results suggest that Kidins220 is an NT vesicle membrane-binding protein and may regulate NT vesicle transport.

PKD1 and PKD2 Regulate Formation of NT Vesicles Containing Kidins220—PKD1 and PKD2, but not PKD3, specifically regulate the surface localization of Kidins220 in cells of neural origin, such as primary cortical neurons and PC12 cells (23). We next compared the intracellular localization of PKD1, PKD2, and Kidins220 utilizing a BON cell line expressing GFP-tagged wild type PKD1 or transiently transfected BON cells with GST-tagged wild type PKD2. In BON cells overexpressing GFP-PKD1WT (Fig. 5A, left panel), Kidins220 was normally expressed in the ends of the processes (Fig. 5A, middle panel). Colocalization of PKD1WT and Kidins220 was not noted (right panel). Similar to the endogenous PKD1 (Fig. 2, left panel), the GFP-tagged PKD1WT also formed large vesicle-like structures in the presence of PMA (Fig. 5B, left panel). Kidins220 formed large vesicle-like structures upon PMA treatment (Fig. 5B, middle panel). Interestingly, Kidins220 remained in the same vesicles as PKD1 (Fig. 5B, right panel). GST-PKD2WT (Fig. 5C, panel a) and Kidins220 (panel b) displayed similar vesicle-like structures formed after PMA treatment (panels c and d). We
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BON cells were treated with various concentrations of PMA for 30 min and Western blot performed. As shown in Fig. 6A, phosphorylation of Kidins220 was increased by PMA treatment in a concentration-dependent fashion (Fig. 6A, top panel). PKD2 phosphorylation was also detected (middle panel), and β-actin was utilized as a loading control (lower panel). To investigate the involvement of Kidins220 in PMA-stimulated NT secretion, BON cells were transfected with two Kidins220 shRNAs and then treated with PMA; NT secretion was measured. PMA-stimulated NT secretion was dramatically decreased by both shRNAs (Fig. 6B, top panel). Western blot demonstrates the attenuation of Kidins220 protein expression (Fig. 6B, bottom panel). To determine whether Kidins220 also regulates NT secretion mediated by physiologic agonists (e.g. the hormone BBS), we performed transient transfections of BON/GFP-GRPR cells with the Kidins220 shRNAs (sh1 and sh2). BBS-stimulated NT secretion was significantly decreased in BON/GFP-GRPR cells transfected with both Kidins220 shRNAs compared with BON/GFP-GRPR cells transfected with control vector in the presence of BBS (Fig. 6C). Taken together, we demonstrate Kidins220 as an important molecule in NT secretion regulated by both pharmacologic and physiologic stimulators.

**DISCUSSION**

Overexpression of kinase-dead PKD isoforms, including PKD1, PKD2, and PKD3, causes TGN tabulation and therefore affects the transport from TGN to the cell surface (24). Previously, we demonstrated that PKD1 positively regulates stimulated NT secretion in a novel human endocrine cell line, BON. In the present study, we further determined that the PKD protein substrate Kidins220 mediates NT secretion and provide evidence for the involvement of PKD/Kidins220 pathway in the regulation of NT hormone secretion. We also extended our previous findings and show that the PKD2 isoform is involved in this process, further confirming the importance of PKD proteins in the regulation of NT release.

PKD proteins, especially PKD1, have been implicated in protein secretion in certain cell types. A single PKD gene has been identified in Drosophila melanogaster, with PKD protein detected in a distinct punctate pattern in secretory tissues, suggesting that PKD plays a role in secretory transport (25). Our study showing overexpression of kinase-dead PKD2 abrogated stimulated NT secretion further demonstrates that the PKD proteins play important roles in hormone secretion in endocrine cells. Consistent with our results, overexpression of the constitutively active PKD1 construct, PKD-ΔPH, increased angiotensin II-mediated cortisol and aldosterone secretion.

FIGURE 6. Kidins220 mediates both PMA- and BBS-stimulated NT secretion. A, BON cells were treated with increasing concentrations of PMA for 30 min and Western blot performed. Experiments were performed at least three times. B, BON cells transiently transfected with Kidins220 shRNA1 (sh1) or shRNA2 (sh2). 24 h after transfection, cells were treated with PMA (10 nM) for 30 min and NT secretion measured (top panel). (*, p < 0.05 versus control vector; †, p < 0.05 versus control vector plus PMA; n = 6). The reduction of Kidins220 expression was shown by Western blot (bottom panel). Experiments were performed at least three times. C, BON/GRP-GRPR cells were transiently transfected with Kidins220 shRNA1 and shRNA2. Cells were treated for 1 h with BBS (10 nM) 24 h after transfection and NT secretion measured (top panel). (*, p < 0.05 versus control vector; †, p < 0.05 versus control vector plus BBS; n = 6). Western blot analysis demonstrates the decreased expression of Kidins220 (bottom panel). Experiments were performed at least three times.

also established BON cells expressing GFP-tagged kinase-dead PKD1 (Fig. 5D, panel a) and performed Kidins220 staining (panel b) and confocal microscopic analysis. Surprisingly, Kidins220 was not identified in the cells overexpressing the kinase-dead PKD1 (Fig. 5D, panel d, arrows), whereas the normal Kidins220 expression was clearly noted in the non-transfected cells (panel d, arrowheads). Moreover, in cells expressing the kinase-dead PKD1 (Fig. 5E, panel a), NT vesicles (panel b) were not detected (panel d, arrows), whereas NT vesicles were noted in untransfected cells (panel d, arrowheads). These results further suggest that PKD proteins regulate formation of NT vesicles containing Kidins220. To further prove whether Kidins220 regulates the formation of NT vesicles, we established the inducible stable cell line, BON/pLenti6/TR-Kidins220 shRNA, in which the knock down of Kidins220 is induced with the addition of doxycycline. The BON/pLenti6/TR cells were grown in the presence or absence of doxycycline (1 μg/ml) for 48 h. Cells were either lysed for Western blot analysis of Kidins220 expression or immunostained for expression of NT. The results from Western blotting showed the expression of endogenous Kidins220 was significantly decreased in the cells treated with doxycycline; the normal expression pattern of NT was not altered in cells with knock down of Kidins220 compared with the control cells (data not shown). This result suggests that Kidins220 regulates secretory processes after the NT vesicles are formed, such as the fusion or the transport to the plasma membrane, whereas PKD1 or PKD2 regulates the transport or formation of NT vesicles after the Golgi.

**Kidins220 Mediates Stimulated NT Release**—We next determined whether Kidins220 was functionally involved in NT secretion. For this purpose, we first analyzed the activation of Kidins220 using the anti-phospho Kidins220 (S919) antibody. Kidins220 is phosphorylated by PKD at serine 919 in vivo (17).
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from H295R cells, a human adrenocortical cell line (26). Chang et al. (27) demonstrated that PKD1-specific shRNA significantly decreased angiotensin II-induced aldosterone secretion in H295R cells. The D2 dopamine receptor was down-regulated in patients with aldosterone-producing adenomas, and, furthermore, down-regulation of the D2 dopamine receptor in aldosterone-producing adenomas increased PKD1 activity and led to overproduction of aldosterone in affected patients (27). Also, PKD1 appears to be involved in insulin secretion stimulated by PMA or BBS in clonal β-cells (HIT-T15) (28).

Kidins220 was recently cloned and identified in PC12 cells (23), a rat pheochromocytoma cell line that secretes catecholamines and other peptides (29), as the first physiologic substrate and binding protein for PKD1 and PKD2, but not PKD3. PKD regulates Kidins220 transport from the TGN to the plasma membrane (23). Endogenous Kidins220 was noted at the plasma membrane and in small punctate vesicles in cells overexpressing the GFP-tagged wild type PKD1, and when kinase-dead GFP-PKD1 was expressed, Kidins220 was mainly found in intracellular clusters, largely colocalizing with the kinase-dead PKD1 (23). We identified Kidins220 expression at the ends of the processes in quiescent BON cells in an expression pattern similar to that of PKD1, PKD2, and NT. In contrast, in cells overexpressing kinase-dead PKD1, endogenous Kidins220 and NT vesicles were not detected, whereas NT vesicle expression pattern was not altered in cells with knock down of Kidins220. Previously, it was demonstrated that the inactive PKD affects the formation of vesicles from Golgi (11–13). Our study suggests that the kinase-dead PKD1 may regulate NT secretion by abrogating the formation of NT vesicles that are Kidins220-positive, whereas Kidins220 is likely more involved in the secretory processes after vesicle formation. Bracale et al. (30) proposed a model in which the kinesin-1 mediated transport of Kidins220-positive carriers to neurite tips and speculated that Kidins220 might regulate the cellular response to neurotrophic stimuli in PC12 cells. These findings further support our results, suggesting that Kidins220 regulates the transport of NT vesicles to the plasma membrane.

Activation of PKD and Kidins220 is important to lipid rafts in PC12 cells, rat primary cortical neurons, and brain synaptosomes (31). Kidins220 concentrates at the tip of neurites upon differentiation (17) and is a downstream effector of neurotrophins and ephrins (32). These findings suggest that Kidins220 may play a role in the process of neuronal differentiation (33). Besides neurons, Kidins220 is also expressed in monocyte-derived and in peripheral blood immature dendritic cells (34). Although recent studies demonstrate the role of Kidins220 in neurotrophin responses (33, 35, 36), the biological function of this protein remains to be extensively investigated. By suppressing endogenous Kidins220 expression using shRNA, we show that Kidins220 regulates NT hormone secretion mediated by PKD1 and PKD2 in a human endocrine cell line, BON. Our findings offer new insights regarding the role of Kidins220 in the PKD signaling pathway.

In conclusion, we have demonstrated that PKD1, PKD2, and their substrate Kidins220 are crucial regulators of NT secretion. Our findings have important clinical implications in the better understanding of signaling molecules responsible for the secretion of hormones and amines from carcinoid tumors that can lead to devastating systemic sequelae in patients with this disease. Finally, because NT plays important roles in inflammation and in the growth of certain cancers that possess NT receptors, the PKD/Kidins220 pathway may serve as a novel target controlling inflammation and tumor growth.

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